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Synthesis and in vitro Inhibition Properties of Oligonucleotide Conjugates Carrying Amphipathic Proline-Rich Peptide Derivatives of the Sweet Arrow Peptide (SAP)

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Abstract A series of derivatives of the amphipathic proline-rich sweet arrow peptide (SAP) were covalently linked to antisense oligonucleotides designed to inhibit *Renilla* luciferase gene. Oligonucleotide-peptide conjugates carrying lysine (Lys) and ornithine (Orn) residues were prepared using the stepwise approach by assembling first the peptide sequence followed by the assembly of the DNA molecule. The resulting Lys, Orn-conjugates were transformed to the corresponding arginine (Arg) and homoarginine (HArg) oligonucleotide-peptide conjugates by reaction with *O*-methylisourea. The introduction of the SAP peptide at 3'-termini of a phosphorothioate oligonucleotide did not affect the ability to inhibit gene expression when transfected with lipofectamine. But, these conjugates were not able to enter cells without transfecting agent. Further studies using SAP peptide as a transfection agent showed promising results for the conjugates carrying the ornithine SAP peptide. All conjugates showed high duplex stabilities.

Keywords

Antisense oligonucleotides, phosphorothioate, luciferase, oligonucleotide-peptide conjugates, sweet arrow peptide, cellular uptake

Introduction

Cell penetrating peptides (CPPs) are considered to be promising delivery agents because they efficiently translocate across the plasma membrane being able to enter in the cells [1, 2]. These CPPs contain domains of less than 20 amino acids and are highly rich in basic residues. This process of translocation across the cell membrane was demonstrated for the first time when the 86-mer trans-activating transcriptional activator, Tat protein, was internalized by cells [3-5]. Since then, researchers have been able to synthesize different and novel peptides or CPPs demonstrating this property of translocation; for instance, transportan [6], synthetic polyarginines [7] or TAT peptide [8]. To date, many modifications have been made in order to synthesize and optimize new CPPs as cellular delivery agents and introduce a variety of intracellular cargoes that have been transported by CPPs like liposomes [9], polymers [10], DNA [11] or nanoparticles [12-14].

Recently, Giralt *et al*. described a new family of amphipathic peptides showing moderate ability to cross the cell membrane in addition to the absence of a cytotoxic effect at high concentrations [12-17]. This proline-rich peptide, called *sweet arrow peptide,* (SAP) whose sequence is $(ValXLeuProProPro)_3$ (where X = His, Arg or Lys (K), respectively) is made up of 50% proline residues, is water-soluble and contains three positively charged X residues favoring interaction with phospholipids anionic polar heads. After various cellular uptake experiments, it was found that two different SAP peptide families (with X = Arg and Lys) could translocate human cell membranes. The positively charged and the amphipathic characteristics of SAP peptides may be of interest for the delivery of negatively charged oligonucleotides in gene silencing experiments.

Oligonucleotides (ODNs) are important biomolecules involved in gene therapy [18, 19]. However, the activity of these compounds *in vivo* are compromised due to several factors like low stability against exonucleases, endonucleases present in serum [20]. Furthermore, delivery into cells is one of the bottlenecks encountered during the use of oligonucleotides in gene therapy. This is primarily due the fact that these compounds are large and negatively charged.

As a part of our ongoing interest in the development of chemically modified DNA [21] and siRNAs [22- 24] to inhibit gene expression, we became interested on the use of CPP for improving cellular uptake of oligonucleotides conjugates. Some authors have already described the attachment of use of CCP for delivery of

oligonucleotides [25]. Although the most common method for the use of CPP for tranfection of oliognucleotides is by using complexes (polyplexes and lipoplexes) thereby taking advantage of the electrostatic interaction of charged oligonucleotides [25], their stabilities could be compromised. In order to obtain more stable complexes and enhance resistance to the nuclease degradation of oligonucleotides, the use of a covalent linkage between CPPs and oligonucleotides has become an interesting alternative in overcoming the use of non-covalent methods which also has obtained promising biological results [26]. However, from a synthetic point of view, combining oligonucleotide chemistry with peptide chemistry covalently proves to be challenging. Surprisingly, the use of CPP-based delivery of antisense oligonucleotides has few references in literature [26-28]. Related to this field, promising results in nuclear delivery efficiencies were achieved for phosphorodiamidate morpholino oligomers (PMOs) that were conjugated to different CPPs such as Tat48-60 (Tat), oligoarginine (R_9F_2) and the 6-aminohexanoic-spaced oligoarginine ((R-Ahx-R)$_4$), obtaining better transfection efficiencies for the last conjugate [28].

In the present study, we looked into the possibility of obtaining new CPP-oligonucleotide conjugates combining the proline-rich SAP peptides with antisense oligonucleotides. Recently, our research group has succesfully used this strategy with the synthesis of several CPPs linked to oligonucleotides through the stepwise approach [21]. In this study, we will describe on the conjugation of amphipathic proline-rich SAP peptides [(Val**X**LeuProProPro)$_3$, where X = Lys, Ornithine (Orn), Homo-arginine (HArg) and Arginine (Arg), respectively] with DNA sequences using this covalent strategy where spacers of different lengths make up the formation of a covalent conjugate between the cargo and the 3'-termini of the oligonucleotides. This strategy was used to obtain an oligonucleotide phosphorothioate designed to inhibit the *Renilla* luciferase gene [21, 29] carrying the aformentioned SAP peptides and the ability of these conjugates to inhibit gene expression was also evaluated in cells. The use of proline-rich peptides as non-viral vectors in cell transfection of plasmid DNA on solid lipid nanoparticles has been recently reported [30]; however to the best of our knowledge, it would be the first time that SAP peptide in combination of antisense oligonucleotides in both covalent and electrostatic aproaches is described for inhibition of gene expression.

Experimental section

Materials and methods

Protected amino acids were obtained from Novabiochem, Merck Chemicals (UK), and Bachem Gmbh (Germany). Poly(ethyleneglycol)-polystyrene resin (PEG-PS) was purchased from PerSeptive Biosystems, Inc. (USA). Coupling reagents: Benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) were purchased from Sigma-Aldrich (USA) and 1-hydroxybenzotriazole (HOBt) were purchased from Albatros Chem Inc., Montreal, Canada. Solvents: Solvents for peptide synthesis and RP-HPLC equipment were obtained from Carlo Erba (Spain). Solvents for oligonucleotide synthesis were purchased from Applied Biosystems (USA). Trifluoroacetic acid (TFA), dimethylformamide (DMF), piperidine and *N*,*N*-diisopropylethylamine (DIEA) were purchased from Sigma-Aldrich (USA); dichloromethane (DCM) was purchased from Panreac (Spain). All commercial reagents and solvents were used as received. Semipreparative RP-HPLC was performed on a Waters (Milford, MA) chromatography system using Symmetry C_{18} (3 × 10 cm, 5 μm) columns. Analytical RP-HPLC was performed using a XBridge OST C18 2.5 μm column. Compounds were detected by UV absorption at 260 nm. Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (Applied Biosystems, Framingham). Two different matrix were used: THAP (2,4,6-trihydroxiacetophenone: 10 mg/mL in ACN:H_2O 1:1 v/v, 0.1% TFA); CA (ammonium citrate: 50 mg/mL in H_2O and SA (sinapinic acid: 10 mg/mL in ACN:H_2O 1:1 v/v, 0.1% TFA). HPLC conditions: Conditions for semipreparative HPLC: HPLC solutions were solvent A: 5% acetonitrile (ACN) in 100 mM triethylammonium acetate (TEAA), pH 6.5 and solvent B: 70 % ACN in 100 mM triethylammonium acetate (pH 6.5). Column: PRP-1 (Hamilton) 250x 10 mm. Flow rate 3 ml/min linear gradient from 15 to 100% in B (*DMT on*) and 0 to 50% in B (*DMT off*) were used with UV detection at 260 nm. Conditions for analytical HPLC: HPLC solutions were solvent A: 5% acetonitrile (ACN) in 100 mM triethylammonium acetate (TEAA), pH 6.5 and solvent B: 70 % ACN in 100 mM triethylammonium acetate (pH 6.5). Column: XBridge OST C18 2.5 μm. Flow rate 1ml/min linear gradient from 0 to 50% in B (*DMT off*) were used with UV detection at 260 nm.

Synthesis of SAP-oligonucleotide conjugates

Solid support **3** was treated with DCM (4 x 30 sec); TFA:DCM (2:3, v/v; 1 x 1 min; 1 x 30 min); DCM (4 x 30 sec); DIPEA:DCM (1:19, v/v; 3 x 1 min) and DCM (4 x 30 sec). Then, the modified succinate unit was reacted with solid support **3** using the following mixture: PyBOP (5.0 eq), DIPEA (10.0 eq), acid (5.0 eq) for 2 hours at room temperature. This mixture was previously pre-activated in DMF and introduced

later on the solid support. The solid support was washed with DMF (4 x 30 sec), DCM (4 x 30 sec), DMF (4 x 30 sec), Ac_2O:DIPEA (500 µL : 900 µL) in DMF (10 min); DMF (4 x 30 sec) and DCM (4 x 30 sec). Then, the solid support was split into two parts, and each one was washed with DCM (4 x 30 sec) and treated with TFA:DCM (2:3, v/v; 1 x 1 min; 1 x 30 min). Solid supports were washed with DCM (4 x 30 sec); DIPEA:DCM (1:19, v/v; 3 x 1 min) and DCM (4 x 30 sec).

Route A: The peptide with a *N*-terminal Boc (Val**Lys**LeuProProPro)$_3$ was prepared by standard "Boc-Chemistry" solid-phase protocols yielding the solid-support **4**. The side chain of lysine was protected with Fmoc group

Route B: The peptide with *N*-terminal Boc (Val**Orn**LeuProProPro)$_3$ was prepared by standard "Boc-Chemistry" solid-phase protocols yielding the solid-support **7**. The side chain of ornithine was protected with Fmoc group.

Then, the *N*-Boc group from resins **4** and **7** were deprotected with a mixture of TFA:DCM (2:3, v/v; 1 x 1 min; 1 x 30 min) and subsequently washed with DCM (4 x 30 sec); DIPEA:DCM (1:19, v/v; 3 x 1 min) and DCM (4 x 30 sec). The resins were split into two parts and trityl derivates **10-12** were introduced in using the same method as the respective amino acids (PyBOP, HOBt, DIPEA in DMF at room temperature). After an acetylation reaction, we obtained the desired Trityl-O-SAP peptides **5** and **8**. The functionalization of the resulting trityl-peptide solid supports was determined by measuring the absorbance of the trityl cation released from the solid-support upon acid treatment.

Then, trityl-peptide solid supports **5** and **8** were transferred directly to a DNA synthesizer (*DMT on*). After assembling the corresponding sequences (*Dickerson-Drew* dodecamer and phosphorothioate oligonucleotide), the resulting solid supports were treated with ammonia solution (32%) and the corresponding modified oligonucleotides were desalted (Sephadex, NAP-10) and purified by semi-preparative reverse phase HPLC. DMT group was removed by a 30 min treatment with 80% acetic acid and removal of the acetic acid by extraction with ether. Finally, the desired oligonucleotide-peptide conjugates **6** and **9** were obtained after desalting (Sephadex, NAP-10). SAP- antisense phosphorothioate conjugates **6c** and **9c** were obtained by DNA precipitation protocols (see below) instead of using Sephadex.

DNA precipitation protocols

DNA conjugates were transfered to a container where it fills one fourth the total volume (a 500 μL tube should have no more than 125 μL of DNA). We added one tenth volume of sodium acetate buffer to equalize ion concentrations. Then, two volumes of cold 100% ethanol were added. Mixtures were cooled at -20 ºC for at least one hour. Finally, we centrifugated the samples for 15 minutes at 4 ºC in a microcentrifuge and remove supernatants. We evaporated the remaining ethanol in a 37 ºC water bath.

Introduction of the guanidinium groups.

SAP-oligonucleotide conjugates **6** and **9** (1.5 OD, approx.) were treated with a solution (125 μL) and were prepared as follows: *O*-methylisourea chloride (100 mg) in water (100 μL) and ammonia (32%; 125 μL). Reaction mixtures were heated at 55 ºC overnight. The solvent was then removed and the respective conjugates were desalted (Sephadex, NAP-5). SAP-oligonucleotide conjugates **13** and **14** were obtained and were checked using analytical HPLC.

Thermal denaturation experiments

Optical measurements were carried out on a Jasco V-650 spectrophotometer. Prior to the experiments, the SAP-oligonucleotide conjugates (each at a final concentration of 2.2 μM to 3.7 μM (approx)), were mixed in 50 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0 conditions. A heating–cooling–heating cycle in the 20 to 90 ºC temperature range was applied. T_m values were determined from the maxima of the first derivative plots of absorbance *versus* temperature.

Cell culture

SH-SY5Y cells were cultured at 37 ºC, 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum. Cells were regularly passaged to maintain exponential growth. Twenty-four hours before transfection at 50-80% confluency, cells were trypsinized and diluted 1:5 with fresh medium (1-3 x 10^5 cells/mL) and transferred to 24-well plates (500 μL per

well). Two luciferase plasmids, Firefly luciferase (pGL3) and *Renilla* luciferase (pRL-TK) (Promega), were used as reporter and as control, respectively.

Transfection of SAP-oligonucleotide phosphorothioate conjugates using Lipofectamine 2000.

Renilla and Firefly luciferase vectors (0.1 μg and 1.0 μg per well, respectively), unmodified and modified SAP- oligonucleotide phosphorothioate conjugates (**6c**, **6d**, **9c**, **9d**, **13c** and **14c**, respectively; 60 nM per well), and a scrambled oligonucleotide (**Scr**; 60 nM per well) was transfected into the cells using Lipofectamine 2000 (Invitrogen) at 6.5 μg/ mL as described by the manufacturer for adherent cell lines. The final volume was 600 μL per well. The cells were harvested 24 hours after transfection and lysed using a passive lysis buffer (100 μL per well) according to the instructions from the Dual-Luciferase Reporter Assay System (Promega). The luciferase activities of the samples were measured using a MicroLuma*Plus* LB 96V Luminometer (Berthold Technologies) with a 2 second delay time and an integrate time of 10 seconds. The following volumes were used: 20 μL of sample and 30 μL of each reagent (Luciferase Assay Reagent II and Stop & Glo Reagent). The inhibitory effects generated by SAP-oligonucleotide phosphorothioate conjugates were expressed as normalized ratios between the reporter GL3 (firefly) luciferase gene and the control RL (*Renilla*) luciferase gene.

Transfection of SAP- oligonucleotide phosphorothioate conjugates in the absence of Lipofectamine 2000.

Renilla and Firefly luciferase vectors (0.1 μg and 1.0 μg per well, respectively) were transfected into the cells using Lipofectamine 2000. Cells were incubated with the plasmids for 5 hours. The medium was discarded and the cells were washed with PBS. Then, 500 μL of fresh medium was added to each well. Finally, 100 μL of SAP-oligonucleotide phosphorothioate solutions (164.4 μM, 14.1 μM, 57.3 μM, 15.4 μM, 23.6 μM and 64.8 μM for **6c**, **6d**, **9c**, **9d**, **13c** and **14c**, respectively in PBS) was added to each well (300 nM per well). Thirty-six hours after transfection, cell lysates were prepared and analyzed using the Dual-Luciferase Reporter Assay System as described above.

Transfection of SAP-oligonucleotide phosphorothioate conjugates using SAP peptide as a transfection agent.

Renilla and Firefly luciferase vectors (0.1 µg and 1.0 µg per well, respectively) were transfected and cells were incubated following the same procedure as described before. The medium was discarded and the cells were washed with PBS. Then, 500 µL of fresh medium was added to each well. Then, 100 µL of SAP-oligonucleotide phosphorothioate conjugates with SAP peptide forming polyplexes in PBS were added to each well (150 nM and 300 nM per well) which were previously incubated at 37 ºC for 45 minutes. Thirty-six hours after transfection, cell lysates were prepared and analyzed using the Dual-Luciferase Reporter Assay System as described above.

Transfection of unmodified oligonucleotide phosphorothioate and SAP peptide.

Renilla and Firefly luciferase vectors (0.1 µg and 1.0 µg per well, respectively) were transfected and cells were incubated following the same procedure as described before. The medium was discarded and the cells were washed with PBS. Then, 500 µL of fresh medium was added to each well. Then, 100 µL of polyplexes in PBS at different concentrations of SAP peptide (20 µM, 30 µM, 40 µM and 50 µM) were added to each well (150 nM and 300 nM per well).

Gel shift assay

Unmodified oligonucleotide phosphorothioate sequence, **wt** (0.5 µg) was mixed with increasing concentrations of SAP peptide, giving rise to SAP peptide: oligonucleotide molar ratios ranging from 1.2 to 10. Complexes were analyzed by electrophoresis on a 20% polyacrylamide gel at 150V for 5 hours in TBE buffer. Picture were taken in Fujifilm LAS-1000 Intelligent Dark box II using IR LAS-1000 Lite v1.2

Results and discussion

Synthesis of SAP-oligonucleotide conjugates

The conjugation of CPPs to various ODNs can be performed by two different strategies: 1) the *non*-covalent strategy in which peptides are able to condense with DNA or RNA molecules involving formation of complexes [25] and 2) the covalent strategy that involves the formation of a covalent conjugate between the cargo and the carrier peptide. This strategy can offer several advantages for *in vivo* applications such as the advantage of being well-defined molecular entities, obtaining better control of the stoichiometry of the CPP-cargo and possible improvement in the stability of the conjugate.

The strategy followed for the synthesis of SAP-oligonucleotide conjugates is outlined on Figure 1. It is well known that there is a relationship between the lysine ε-amine group and ornithine δ-amine group with their respective homo-arginine (HArg) and arginine (Arg) amino acids. Taking into account the SAP peptide structure $(ValXLeuProProPro)_3$, the stepwise approach was used in order to synthesize the SAP-oligonucleotides conjugates (**1**) in their amine form (where X = lysine (Lys) and ornithine (Orn), respectively) (Fig. 1, Step A). To this end, Boc-amino acids were used and the side chains of lysine and ornithine were protected with the base labile trifluoroacetyl (Tfa) and fluorenylmethoxycarbonyl (Fmoc) groups, respectively. The first amino acid was linked to the solid support by using the 6-aminohexylsuccinyl linker [21]. All together allow the removal of the side chain protecting groups and the liberation of the peptide from the solid support with an ammonia treatment used in oligonucleotide synthesis. However, introducing the three guanidinium groups (Fig. 1, Step B) is not straightforward. It has been described the use of the di-Fmoc-derivative of arginine in a stepwise protocol [31] but this derivative is not commercially available. Post-synthetic approaches have been also described [32, 33]. Recently, our research group reported the synthesis of phosphorothioate conjugates containing different residues of homo-arginines through post-synthetic approach according to the literature [24]. Following this strategy, we were able to introduce the corresponding guanidinium groups (where X'= homo-arginine and arginine, respectively) yielding the respective SAP-oligonucleotide conjugates (**2**) (Figure 1, Step B).

In order to demonstrate our proposed strategy and the stability of the SAP-oligonucleotide conjugates, the self-complementary *Dickerson-Drew* dodecamer [34] sequence (5'-CGCGAATTCGCG-3'; Sequence A) was used as a model sequence. The synthesis of the conjugate **1** began with commercially available poly(ethyleneglycol)-polystyrene (PEG-PS, **3**) (Perseptive Biosystems, 0.19 mmol/gr) as starting material. The first spacer was introduced into the resin and then was split equally into two parts: a lysine residue was introduced (Route A) whereas ornithine was introduced (Route B) thereby obtaining the appropriate peptides **4** and **7** by standard "Boc and Fmoc chemistry" solid-phase protocols whose peptide

sequences were (Val**Lys**LeuProProPro)$_3$ and (Val**Orn**LeuProProPro)$_3$, respectively. The last Boc group was then removed and the resulting free amines were reacted with two spacer-trityl derivatives (**10** and **11),** which were obtained in accordance with the literature [35]. The trityl modified solid-supports (**5** and **8)** were transferred directly to a DNA synthesizer and the respective phosphoramidites were coupled to the self-complementary sequence model 5'-CGCGAATTCGCG-3' (*Dickerson-Drew* dodecamer) at the 3'-position. The solid supports were then cleaved with an ammonia solution (32%) at 55 ºC overnight and the corresponding SAP-oligonucleotide conjugates were obtained in their amine form. The conjugates were purified according to *DMT on* based protocols (Fig. 2) and the resulting SAP-oligonucleotide conjugates (**6a**, **6b**, **9a** and **9b)** were fully characterized by MALDI-TOF mass spectrometry (Supplementary material, Table 1S).

The modification of the amine group to the guanidinium moiety in the peptide sequence was carried out using a post-synthetic modification approach using a freshly prepared solution of *O*-methylisourea, yielding the expected SAP-guanidino conjugates **13a**, **13b**, **14a** and **14b**, respectively (Fig. 3). The guanidynilation reaction was quantitative yielding the respective conjugates in pure form and without further purification. The resulting conjugates were confirmed using analytical HPLC chromatography and MALDI-TOF mass spectrometry (Supplementary material, Table 1S).

Synthesis of SAP-oligonucleotide phosphorothioate conjugates and biological evaluation

Next, according to the strategy described before, SAP-oligonucleotide conjugates (**6c**, **6d**, **9c**, **9d**, **13c**, **14c**) were synthesized and evaluated in gene silencing experiments. We recently described an oligonucleotide phosphorothioate sequence that is active in inhibiting *Renilla* luciferase gene (5'-AGGTCTTGTTTCCTTTGC-3'; Sequence B) when is linked to polylysine and polyhomoarginine peptides at 3'-termini [21]. Thus, a series of SAP peptides ((ValXLeuProProPro)$_3$ with X = Lys, Orn, HArg and Arg) were covalently combined with the aforementioned *Renilla* phosphorothioate sequence which was designed and evaluated as potential antisense oligonucleotides for inhibiting the *Renilla* luciferase gene. SAP-phophorothioate conjugates (**6c**, **6d**, **9c**, **9d**, **13c** and **14c**, respectively) were obtained from the previous solid-suports **5b** and **8b** following the stepwise solid-phase and post-synthetic modification methods described for the *Dikerson-Drew* dodecamer. All conjugates were fully characterized by MALDI-TOF mass spectrometry (see Supplementary material, Table 1S), except for **9d** in which we were not able to detect the expected mass

by spectrometry. In order to determine whether we had conjugate **9d** in our hands, all conjugates were analyzed by denaturing polyacrylamide gel electrophoresis (PAGE) in 20% polyacrylamide, 8M urea (see Supplementary material, Fig. 2S). As expected, the conjugate **9d** showed similar retention than the rest of the SAP-antisense phosphorothioate conjugates.

The inhibitory properties of the resulting SAP-antisense phosphorothioate conjugates were analyzed in SH-SY5Y cells. The cells were co-transfected with two luciferase plasmids (*Renilla* and firefly; target and internal control, respectively), SAP-antisense phosphorothioate conjugates (**6c**, **6d**, **9c**, **9d**, **13c** and **14c** at 60 nM), a scrambled sequence (**Scr**) and the same scramble sequence conjugated with SAP peptide containing ornithine using commercial Lipofectamine 2000. We also included the antisense phosphorothioate sequence without SAP peptide (**wt**). The luciferase activities were measured by using a luminometer twenty-two hours after transfection. The results, showing *Renilla* luciferase activity normalized to firefly luciferase, are shown on Fig. 4. Interestingly, all SAP-oligonucleotide conjugates demonstrated gene knockdown activities (71%, 62 %, 81%, 82%, 77% and 81% for **6c**, **6d**, **9c**, **9d**, **13c** and **14c**, respectively) similar to the phosphorothioate sequence with no peptide (82% for **wt**). These results confirm that the SAP peptide as a modification does not disrupt antisense activity nor does it interfere with cellular activity.

Next, we carried out transfection experiments in the absence of lipofectamine in order to know the capacity of our conjugates to impart cellular uptake. After incubating the two luciferase plasmids for five hours, the cell medium was discarded and the cells were incubated again with a fresh medium; the SAP-antisense phosphorothioate conjugates (**6c**, **6d**, **9c**, **9d**, **13c** and **14c**) and the phosphorothioate sequence without peptide (**wt**) at 300 nM. The luciferase activities were measured thirty-six hours after transfection. Unfortunately, the conjugates were not able virtually to penetrate SH- SY5Y cells by themselves (3%, 3 %, 3%, 2 %, 1% and 0% knockdown of *Renilla* expression at 300 nM for **6c**, **6d**, **9c**, **9d**, **13c** and **14c**, respectively) (Supplementary material, Fig. 3S).

Transfection efficiencies wih unmodified ASO phosphorothioate and SAP peptide forming complexes

The ability of certain cationic peptides to condense DNA, specifically arginine-rich peptides for improving DNA delivery has been described [36]. The formation of non-covalent complexes between CPPs and oligonucleotides has been reported by several research groups and the complex formation has

been also characterized using different biophysical techniques [37]. Recently, the ability of SAP peptide to translocate human cell membranes has been reported. This property is in agreement with the inherent properties to the amphipathic helix character of the SAP peptide when arginine is present in the peptide sequence thereby being most efficient for cellular uptake process [15, 30]. Furthermore, due to its non-viral origin, amphipathic character and solubility in water, SAP peptide could *a priori* present several advantages as a transfection agent taking advantage of its positive charges.

First, we evaluated the amount of required SAP peptide ($(ValArgLeuProProPro)_3$) in which the unmodified antisense phosphorothioate is able to form complexes. We performed a gel shift assay to evaluate the amount of SAP peptide that efficiently interacted with an unmodified phosphorothioate oligonucleotide, **wt** (Supplementary material, Fig. 1S). Several concentrations of the peptide were added, from 1.2-fold molar excess of the peptide compared with **wt** oligonucleotide to a 10-fold molar excess. We observed that SAP peptide was able to retard completely the oligonucleotide phosphorothioate, **wt** at a molar ratio of 10:1, as shown in Fig. 1S, lane 7.

To assess whether the interaction of the SAP peptide with the oligonucleotide phosphorothioate, as shown in the gel shift assay, was able to impart cellular uptake in the absence of a transfection agent, we performed the experiments described before in SH-SY5Y cells using pGL3 as a reporter gene. The molar ratios tested for gene knockdown were 4:1, 6:1, 8:1 and 10:1 respectively (peptide:antisense oligonucleotide). Interestingly, we observed that the majority of the complexes had gene specific inhibitory properties at 300 nM thereby obtaining promising results (around 40% of inhibition efficacy in all cases), except for the first case (4-fold molar excess of SAP peptide) which its activity was detrimental (Fig. 5). This is not surprising according to the SDS-PAGE where we clearly observed the complex formation using at least 6-fold of peptide. A scramble sequence (**Scr**) was also used in this study as a control to establish that the gene knockdown was specific. This sequence was only complexed with the peptide at a 6:1 M ratio yielding no effect on luciferase expression. Experiments at 150 nM were also performed. As expected, we clearly observed that SAP peptide and unmodified phosphorothioate (**wt**) complexes significantly decreased the luciferase activity in a concentration-dependent manner (see Supplementary material, Fig. 4S).

Transfection efficiencies wih SAP-oligonucleotide phosphorothioate conjugates and SAP peptide forming complexes

Next we studied the use of the SAP peptide as transfecting agent for the SAP-antisense conjugates. To this end, we analyzed the gene knockdown effect in SH-SY5Y cells by using SAP-antisense conjugates (**6c, 6d, 9c, 9d, 13c** and **14c**, respectively) along with SAP peptide acting as a transfecting agent in a 6-fold molar excess (peptide:SAP-antisense conjugate). We subjected the same protocol as described before for unmodified antisense oligonucleotide to perform *in vitro* transfections at 150 nM. Notably, we observed an improvement in the activities inhibition when SAP peptide was used as a transfection agent. The majority of SAP-antisense conjugates in combination with SAP peptide were able to inhibit *Renilla* gene expression thereby slightly improving the activities compared to the experiments previously described without using any transfection agent (11%, 20%, 26%, 27%, 14% and 26% for **6c**, **6d**, **9c**, **9d**, **13c** and **14c**, respectively) (Supplementary material, Fig. 5S-A). Although the majority of the conjugates showed similar activities to those obtained with unmodified antisense:SAP polyplexes, surprisingly, we found that conjugates containing ornithine and arginine in their peptide sequence (**9c**, **9d** and **14c**) had slightly better transfection efficiencies than conjugates containing lysine and homo-arginine (**6c**, **6d** and **13c**).

We also evaluated the inhibitory properties of SAP-antisense phosphorothioate conjugates using SAP peptide as a transfection agent at 300 nM and 6:1 molar (peptide: SAP-antisense conjugate) ratio. Surprisingly, only SAP-antisense conjugates containing ornithine (22% and 18% for **9c** and **9d**, respectively) were able to knockdown gene expression (Supplementary material, Fig. 5S-B) whereas the activities in gene-knockdown expression for SAP-antisense conjugates containing lysine, homo-arginine and arginine (**6c**, **6d**, **13c** and **14c**, respectively) were detrimental (see Supplementary material, Fig. 5S). To our surprise, transfections carried out at 300 nM did not keep a typical dose-response behavior compared to the experiments performed at 150 nM as we observed previously with polyplexes containing unmodified antisense oligonucleotide (**wt**). These results might be explained in light of the potential cytotoxic effects imparted by some conjugates in combination with the peptide at high concentrations on the SH-SY5Y cells thereby suggesting that gene knockdown expression might be compromised at 300 nM.

Finally, in view of all these results, we compared the effect of SAP peptide as a transfection agent to lipofectamine in the same gene knockdown experiments and cell line. SAP-antisense oligonucleotide conjugates containing ornithine and arginine (**9c**, **9d** and **14c**) were chosen to examine the corresponding

transfection efficiencies, discarding the rest of the conjugates. As expected, experiments carried out with lipofectamine gave better results in gene knockdown effects than SAP peptide in our hands at both 60 nM and 150 nM (Fig. 6). In this case we also observed that the gene inhibitory properties of the conjugates when SAP peptide is used as transfecting agent were better at lower oligonucleotide concentration (60 nM) than at higher concentration (150 nM). This effect was not seen when transfection was done with Lipofectamine. These data indicates that the formation of efficient transfecting polyplexes of conjugates with the SAP peptide is dependent of the concentration of both peptide and oligonucleotide conjugate and, for some reason, at higher conjugate concentration the polyplexes formed may not be efficient for cell transfection. But, at lower concentration of oligonucleotide conjugate the polyplexes formed are efficient for transfection. Best results were obtained at 60 nM with the ornithine containing conjugates (62% inhibition **9c**, 67% inhibition **9d**). In these conditions the molar ratio between peptide and oligonucleotide conjugate is 6:1 while in the lipofectamine experiments lipofectamine is used at approximately 100 molar excess and reaches 81-82% inhibition.

Thermal denaturation experiments

The duplex stabilities of conjugates **6**, **9**, **13** and **14** (phosphate and phosphorothioate conjugates, respectively) were measured using the following conditions: 50 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0 (Table 1). Under these conditions, all phosphate conjugates (amine and guanidinium series, respectively) showed higher T_m values than the unmodified oligonucleotide (T_m = 44 ºC for the *Dickerson-Drew* dodecamer). The higher stabilities were found in the lysine (ΔTm 12.4, 17.8 ºC) and ornithine (ΔTm 14.4, 14.8 ºC) conjugates. Duplexes carrying homoarginine (ΔTm 10.0, 12.2 ºC) and arginine (ΔTm 15.0, 7.0 ºC) were, in general, less stable. In the case of phosphorothioate conjugates the unmodified antisense oligonucleotide melted at 30.5 ºC; and most of the conjugates had a slight stabilization (ΔTm 1.5-3.4 ºC) except conjugate **9c** that melted at a temperature slightly lower than the unmodified phosphorothioate (T_m = 29.9 ºC) .

Conclusions

We synthesized two different amphipathic rich-proline peptide (SAP) families covalently linked to oligonucleotides using spacers of different lengths between both biomolecules. In general, both SAP-

oligonucleotides and SAP-antisense phosphorothioate conjugates containing lysine in their structure were obtained with a higher yield than the SAP-oligonucleotide conjugates containing ornithine. This difference in reactivity could be attributed to the existing side-reactions consisting of the premature and undesired removal of the Fmoc group caused by a primary amine of sufficient basicity like the amine group of ornithine present in the peptide resin [38]. The introduction of guanidinium groups was carried out using post-synthetic approximation with better results and purity levels.

Studies in the inhibition of gene expression showed that the introduction of the SAP peptide at 3'-position of an antisense oligonucleotide does not affect antisense activity. However, the cell uptake experiments that we carried out with the conjugates were compromised. This lack of activity could be attributed to a poor cellular uptake of SAP conjugates in absence of lipofectamine. Next, we carried out gene-knockdown experiments using SAP peptide itself as a transfection agent due to its advantages as non-viral origin, amphipathic character and the absence of cytotoxic effects at high concentrations. SAP peptide was able to form polyplexes with the corresponding SAP-antisense conjugates, obtaining promising results for SAP-antisense conjugates containing ornithine in their peptide sequence. We also evaluated the effect of complexation between SAP peptide and unmodified antisense phosphorothioate sequence thereby also obtaining promising results of around 40% of gene expression inhibition at 300 nM. This first generation of conjugates complexed with amphipathic SAP peptide represent a promising lead for future optimization in both antisense therapy and RNA interference.

Finally, we measured the thermal stability of duplexes carrying SAP peptides and found there was a remarkable stabilization of the duplex induced by the presence of the positively charged peptide.

Supporting Information

Experimental procedures, HPLC chromatograms, MALDI-TOF mass spectrometry graphs, denaturing and naturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transfection results without using lipofectamine and dose-response experiments.

Acknowledgements

This research was supported by the Spanish Ministry of Education (Grant CTQ2010-20541), the Generalitat de Catalunya (2009/SGR/208), and the Instituto de Salud Carlos III (CB06_01_0019). CIBER-BBN is an initiative funded by the VI National R&D&i Plan *2008-2011, Iniciativa Ingenio 2010, Consolider Program, CIBER Actions* and financed by the Instituto de Salud Carlos III with assistance from the *European Regional Development Fund.* We gratefully acknowledge Dra. Miriam Royo and the Peptide Synthesis Unit from the CIBER-BBN Production of Biomolecules Platform for synthesizing SAP peptide.

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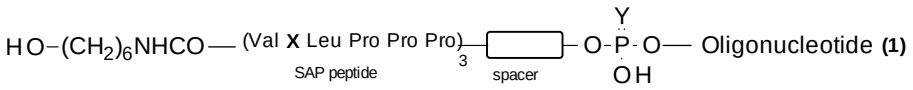
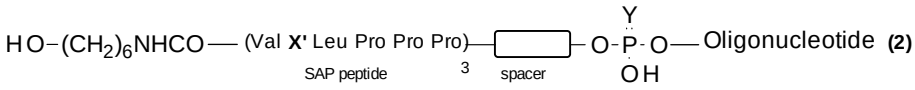
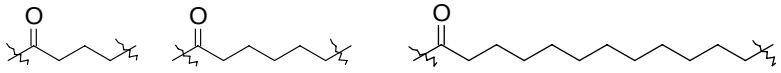
Fig. 1 Strategies for the synthesis of SAP-oligonucleotide conjugates in their amine (1) and guanidinium form (2) using stepwise approach (Step A) or post-synthetic approach (Step B)

1. Step A (stepwise approach)



2. Step B (post-synthetic approach)



spacer = 

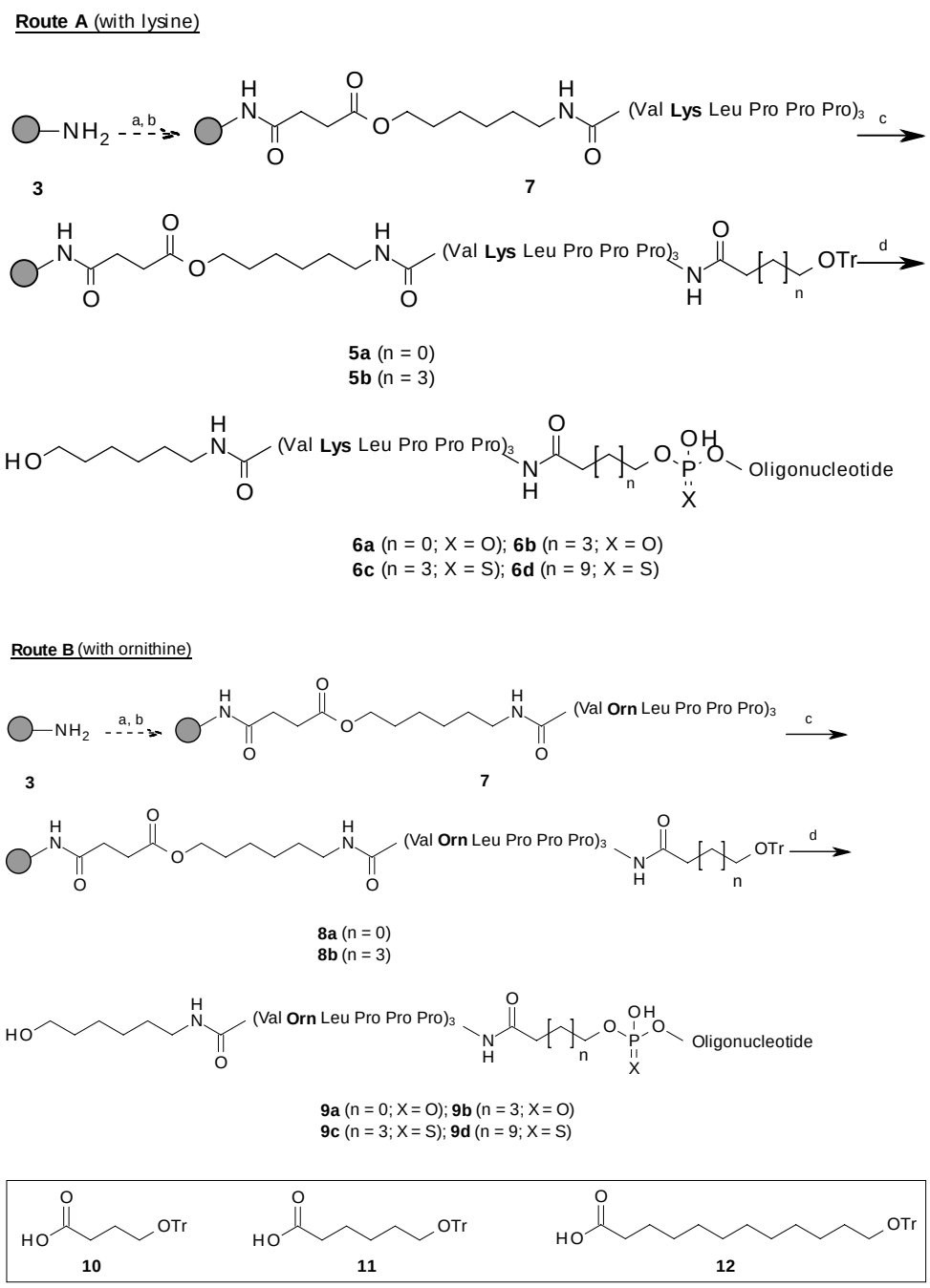
where X = Lysine, Ornithine

X' = Homo-Arginine, Arginine

Y = O, S

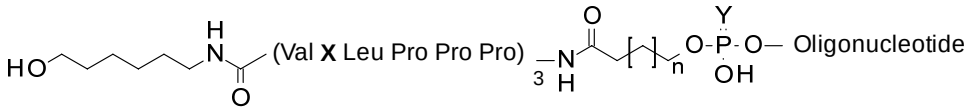
ODN = 5'-CGCGAATTCGCG-3'
5'-AGGTCTTGTTTCCTTTGC-3'

Fig. 2 Stepwise synthesis of SAP-oligonucleotide conjugates containing lysine (**6**) and ornithine (**9**)



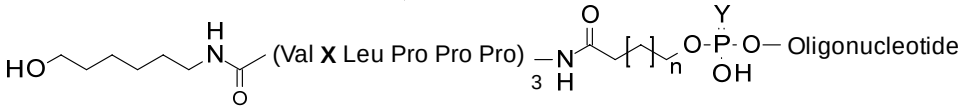
Reagents and Conditions a, b. "Boc-Fmoc chemistry" solid-phase protocols; c. HOBt, PyBOP, DIEA, **10**, **11** or **12**, DMF, r.t., 2h; d. i. NH_3 (32%); ii. DMT on purification; iii. AcOH 80%, 30 min; iv. NAP-10

Fig. 3 Guanydinylation reaction used for the synthesis of SAP-oligonucleotide conjugates **13** and **14**



6a (X = Lys; Y = O; n = 0); **9a** (X = Orn; Y =O; n = 0)
6b (X = Lys; Y = O; n = 3); **9b** (X = Orn; Y = O; n = 3)
6c (X = Lys; Y = S; n = 3); **9c** (X = Orn; Y = S; n = 3)

a



13a (X = HArg; n = 0); **13b** (X = Arg; n = 0); **13c** (X = HArg; Y = S; n = 3)
14a (X = HArg; n = 3); **14b** (X = Arg; n = 3); **14c** (X = Arg; Y = S; n = 3)

Reagents and Conditions: a. i. NH_3(32%), O-methylisourea, H_2O, 55 °C, overnight; ii. Sephadex

Table 1. Melting temperatures of SAP-oligonucleotide conjugates carrying phosphodiester and phosphorothioate linkages.

Sequence (5'->3')	SAP-DNA conjugate	Tm (ΔTm)[a] / ºC
A	**6a**	56.4 (12.4)
A	**6b**	61.8 (17.8)
A	**9a**	58.4 (14.4)
A	**9b**	58.8 (14.8)
A	**13a**	54.0 (10.0)
A	**13b**	56.2 (12.2)
A	**14a**	59.0 (15.0)
A	**14b**	51.0 (7.0)
B	**6c**	32.0 (1.5)
B	**6d**	33.9 (3.4)
B	**9c**	29.9 (-0.6)
B	**9d**	32.5 (2.0)
B	**13c**	35.6 (3.1)
B	**14c**	32.2 (1.7)
A	unmodified	44.0
B	**wt,** unmodified	30.5

n.d. not determined; [a]ΔTm is the difference between the melting temperature of the modified oligonucleotide minus the melting temperature of the corresponding unmodified oligonucleotide; Buffer conditions: 50 mM NaCl, 10 mM; sodium phosphate pH 7.0

Fig. 4 Gene-specific silencing activities for phosphorothiote oligonucleotides (**wt**, **6c**, **6d**, **9c**, **9d**, **13c**, **14c**, **Scr** and **Scr-Orn**) (60 nM per well) targeting the *Renilla* luciferase mRNA expressed in SH-SY5Y cells. Transfection of antisense oligonucleotides was carried out by using Lipofectamine 2000. Two scrambled sequences (**Scr** and **Scr-Orn**) gave no *Renilla* luciferase inhibition.

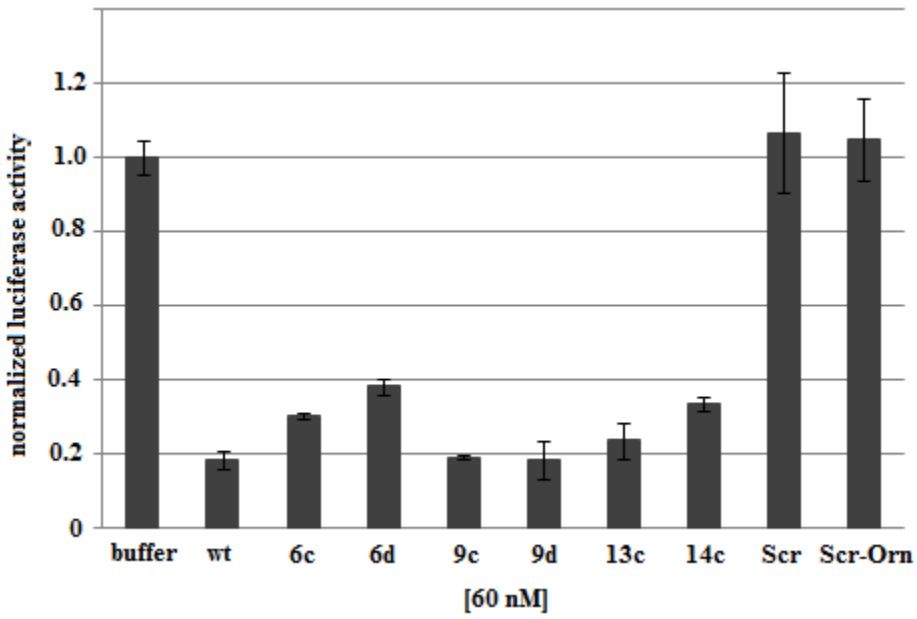


Fig. 5 Gene-specific silencing activities for unmodified phosphorothioate oligonucleotide (**wt**) with and without SAP-peptide (300 nM per well) targeting the *Renilla* luciferase mRNA expressed in SH-SY5Y cells. The molar ratios tested for gene knockdown were 4:1, 6:1, 8:1 and 10:1 respectively (peptide:antisense oligonucleotide). Transfection of antisense oligonucleotide was carried out by using SAP peptide. Scrambled sequence (**Scr**) gave no *Renilla* luciferase inhibition. The bar named **wt** is the result of luciferase activity without using SAP peptide as transfecting agent.

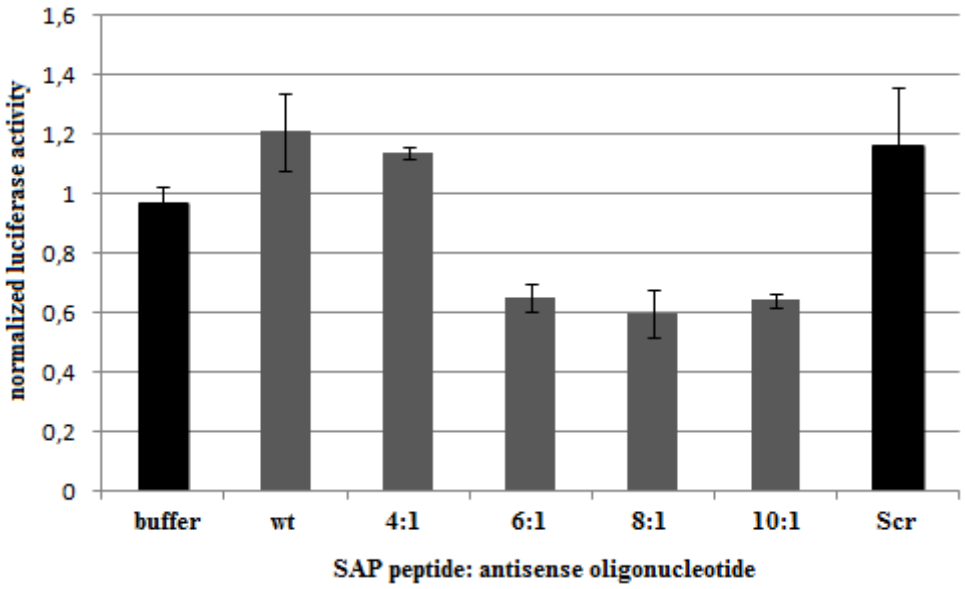
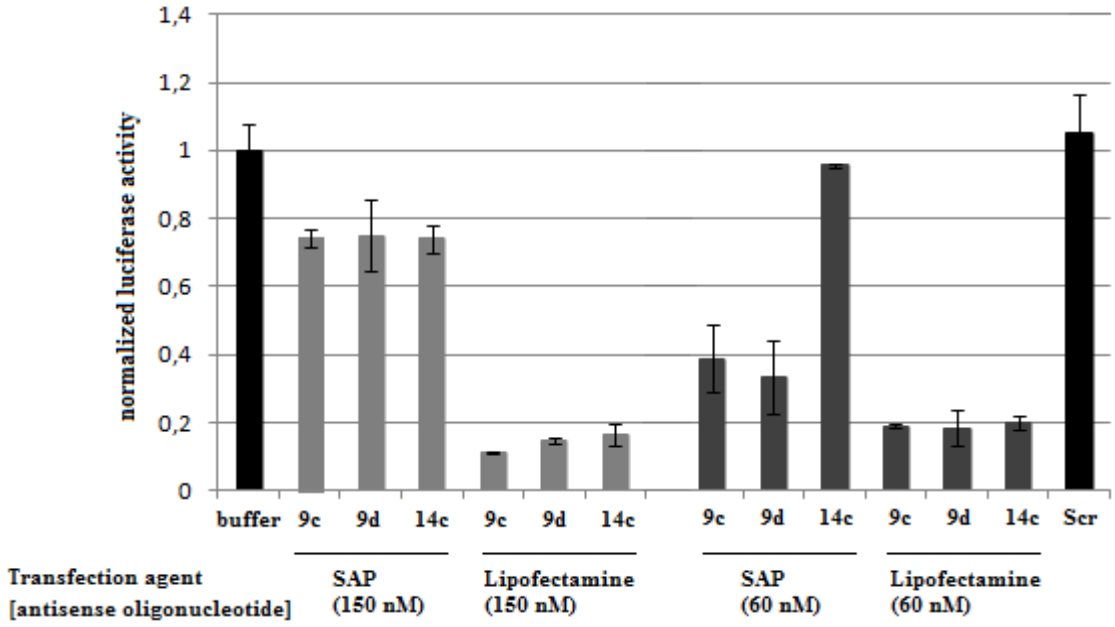


Fig. 6 Gene-specific silencing activities for SAP-antisense oligonucleotide conjugates at 60 nM and 150 nM with lipofectamine and SAP peptide as transfection agents. The peptide/ antisense oligonucleotide molar ratio used was 6:1 targeting the *Renilla* luciferase mRNA expressed in SH-SY5Y cells. A scrambled sequence combined with SAP peptide in a 6:1 M ratio gave no *Renilla* luciferase inhibition (both at 60 nM and 150 nM).



Supplementary Data

Synthesis and *in vitro* Inhibition Properties of Oligonucleotide Conjugates Carrying Amphipathic Proline-rich Peptide Derivatives of the Sweet Arrow Peptide (SAP)

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*e-mail: recgma@cid.csic.es

Summary

Cell culture

Table 1S. MALDI-TOFF mass spectrometry

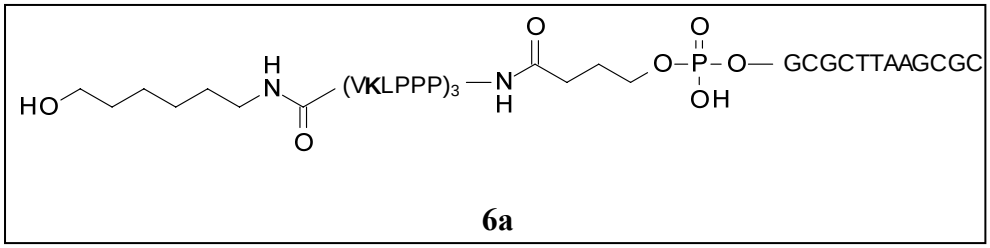
Sequence (5'->3')	SAP-ODN conjugate	MW (calc)	MW (found)
A	6a	5793	5800
A	6b	5821	5824
B	6c	7898	7909
B	6d	8010	8033
A	9a	5750	5752
A	9b	5779	5783
B	9c	7884	5969*
B	9d	7968	n.d.
A	13a	5919	5920
A	13b	5947	5951
B	13c	8046	8020
A	14a	5887	5891
A	14b	5877	5908
B	14c	7995	5926*
B	Scr-Orn	7884	5935*
A	unmodified	n.d	n.d.
B	unmodified	5715	5720

Sequence A (phosphate form): 5'-CGCGAATTCGCG-3'

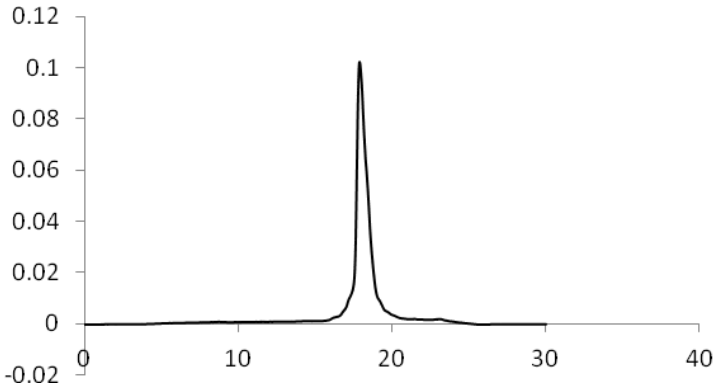
Sequence B (phosphorothioate form): 5'- AGGTCTTGTTTCCTTTGC-3'

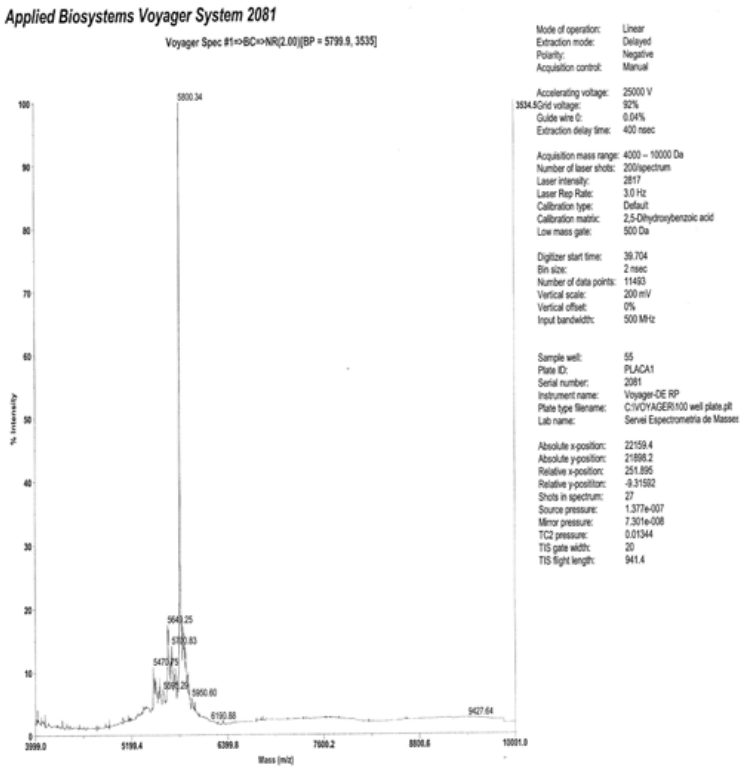
n.d. not determined; *the mass corresponds to the removal of the whole peptide and the two spacers

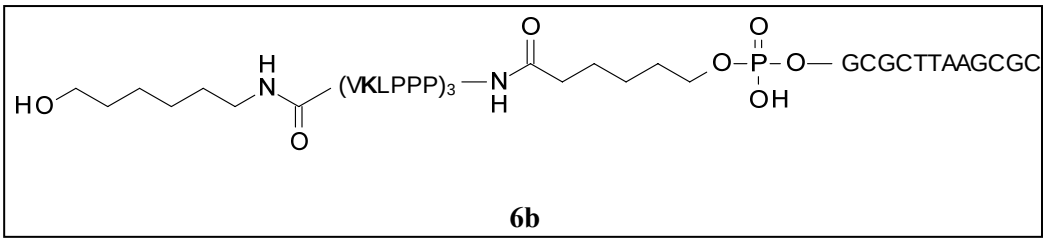
SAP-ODN conjugates. HPLC chromatograms and MALDI-TOFF mass spectrometry spectra



6a






HO
H
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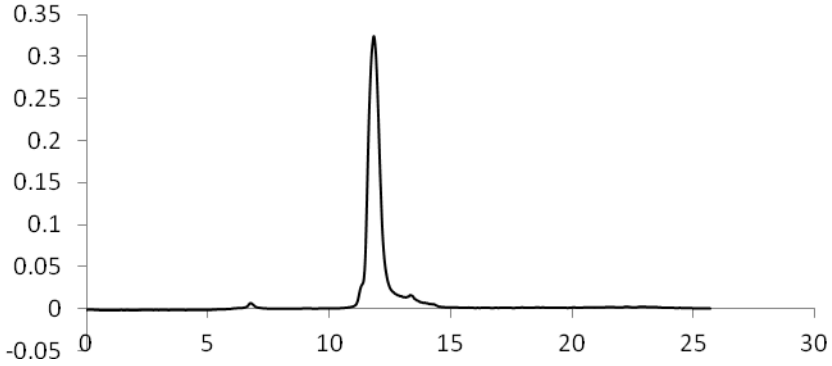
(VKLPPP)3
N
H
O
O
O
P
O
OH
O
GCGCTTAAGCGC

6b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0.1
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0
-0.05
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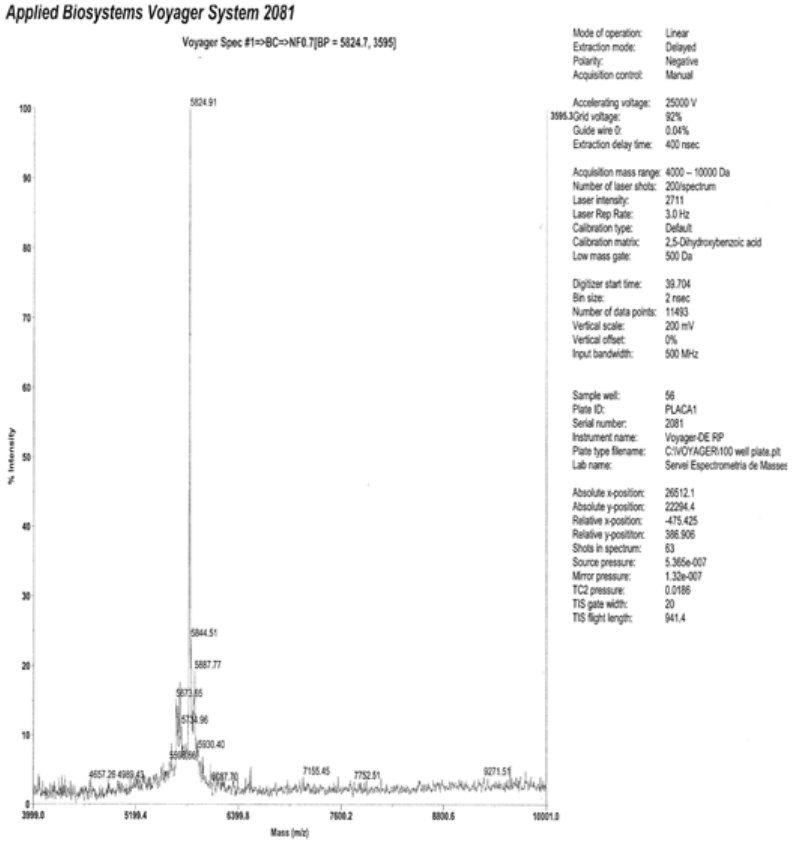
5
10
15
20
25
30


Applied Biosystems Voyager System 2081
Voyager Spec #1=>BC=>NF0.7[BP = 5824.7, 3595]
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Mass (m/z)
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5887.77
5973.85
6134.96
6930.40
7155.45
7752.51
9271.51
Mode of operation: Linear
Extraction mode: Delayed
Polarity: Negative
Acquisition control: Manual
Accelerating voltage: 25000 V
3595.3Grid voltage: 92%
Guide wire 0: 0.04%
Extraction delay time: 400 nsec
Acquisition mass range: 4000 – 10000 Da
Number of laser shots: 200/spectrum
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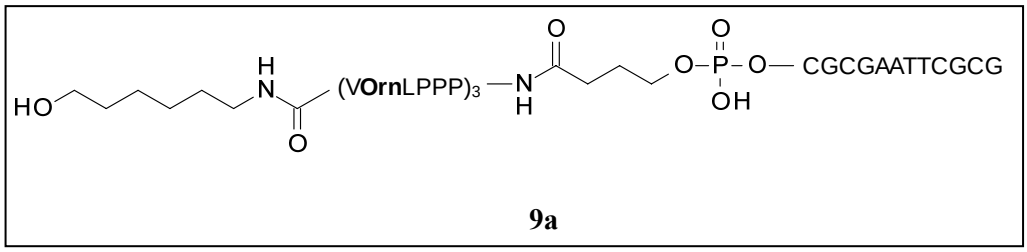
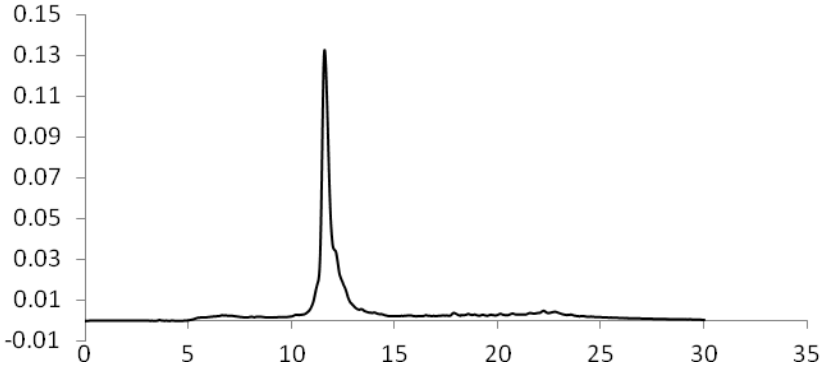
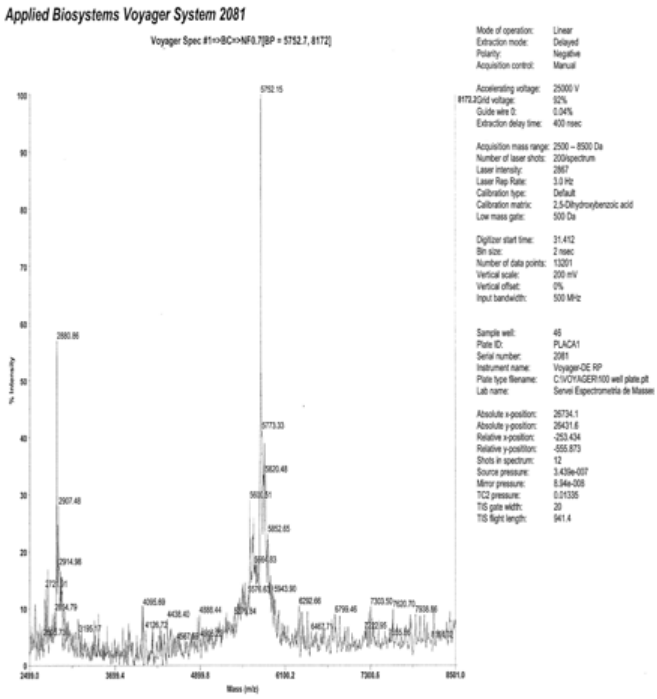
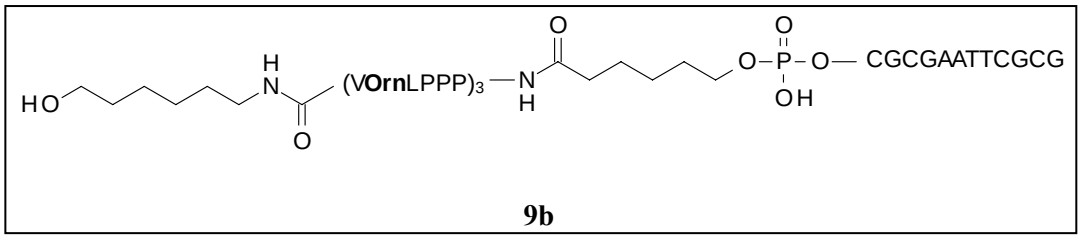
Laser Rep Rate: 3.0 Hz
Calibration type: Default
Calibration matrix: 2,5-Dihydroxybenzoic acid
Low mass gate: 500 Da
Digitizer start time: 39.704
Bin size: 2 nsec
Number of data points: 11493
Vertical scale: 200 mV
Vertical offset: 0%
Input bandwidth: 500 MHz
Sample well: 56
Plate ID: PLACA1
Serial number: 2081
Instrument name: Voyager-DE RP
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: Servei Espectrometria de Masses
Absolute x-position: 26512.1
Absolute y-position: 22294.4
Relative x-position: -475.425
Relative y-posititon: 386.906
Shots in spectrum: 63
Source pressure: 5.365e-007
Mirror pressure: 1.32e-007
TC2 pressure: 0.0186
TIS gate width: 20
TIS flight length: 941.4


HO
H
N
O
(VOrnLPPP)3
N
H
O
O
O
P
O
OH
O
CGCGAATTCGCG

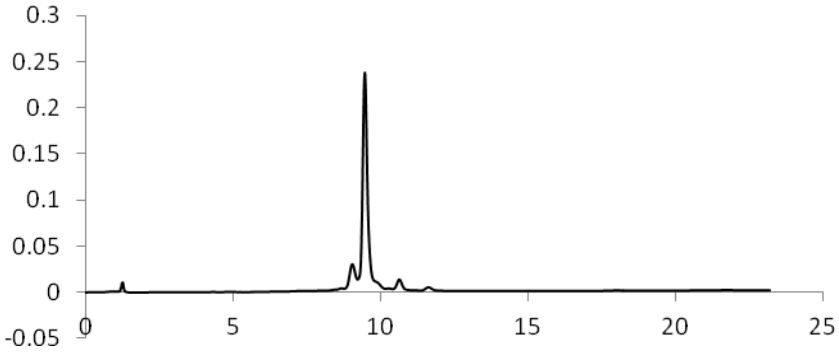
9a

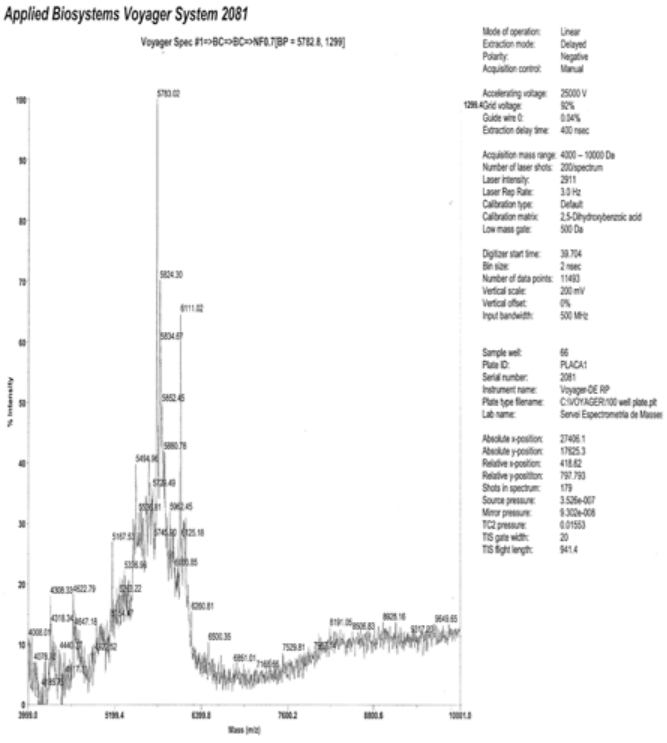

0.15
0.13
0.11
0.09
0.07
0.05
0.03
0.01
-0.01
0
5
10
15
20
25
30
35


Applied Biosystems Voyager System 2081
Voyager Spec #1=>BC=>NF0.7[BP = 5752.7, 8172]
Mode of operation: Linear
Extraction mode: Delayed
Polarity: Negative
Acquisition control: Manual
Accelerating voltage: 25000 V
Grid voltage: 92%
Guide wire 0: 0.04%
Extraction delay time: 400 nsec
Acquisition mass range: 2500 – 8500 Da
Number of laser shots: 200/spectrum
Laser intensity: 2867
Laser Rep Rate: 3.0 Hz
Calibration type: Default
Calibration matrix: 2,5-Dihydroxybenzoic acid
Low mass gate: 500 Da
Digitizer start time: 31.412
Bin size: 2 nsec
Number of data points: 13201
Vertical scale: 200 mV
Vertical offset: 0%
Input bandwidth: 500 MHz
Sample well: 46
Plate ID: PLACA1
Serial number: 2081
Instrument name: Voyager-DE RP
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: Servei Espectrometria de Masses
Absolute x-position: 26734.1
Absolute y-position: 26431.6
Relative x-position: -253.434
Relative y-position: -555.873
Shots in spectrum: 12
Source pressure: 3.439e-007
Mirror pressure: 8.94e-008
TC2 pressure: 0.01335
TIS gate width: 20
TIS flight length: 941.4
5752.15
2880.86
5773.33
2907.48
5820.48
5852.65
2914.98
4095.69
4438.40
4888.44
6292.66
6798.46
7303.50
7820.70
7936.86
Mass (m/z)

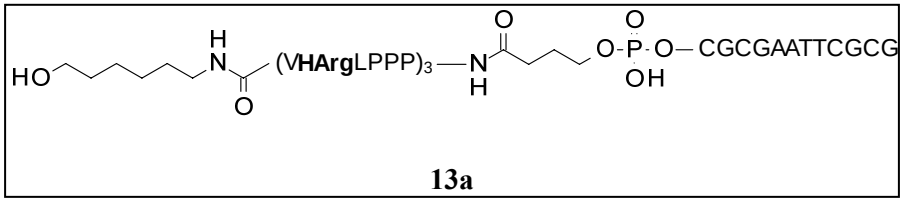


9b

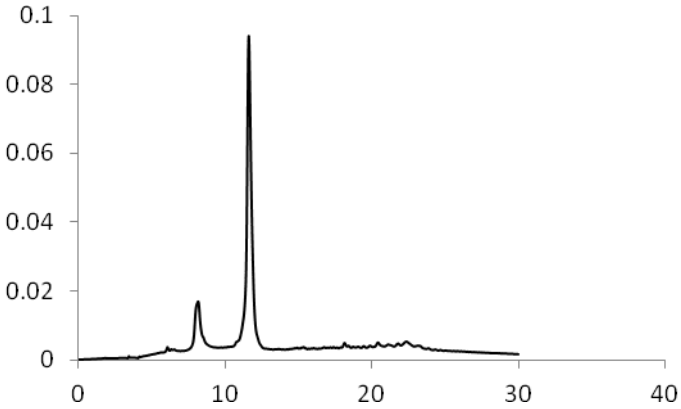


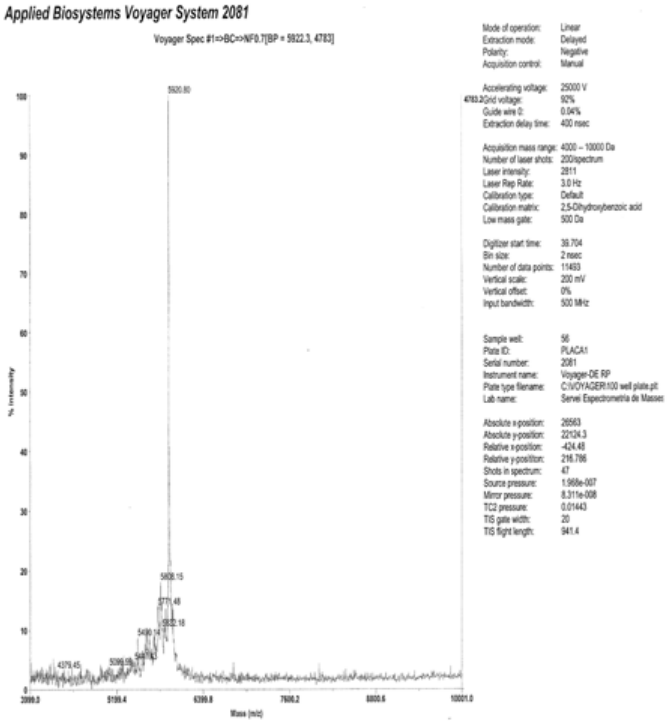


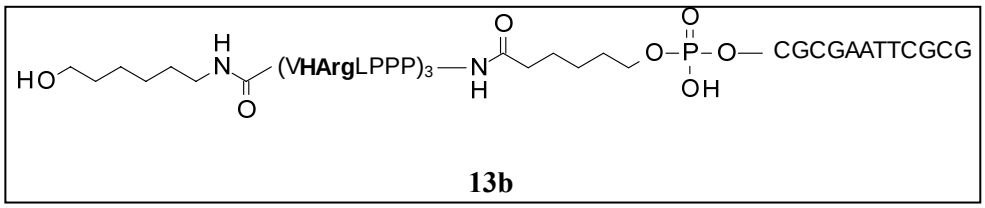
Mode of operation:	Linear
Extraction mode:	Delayed
Polarity:	Negative
Acquisition control:	Manual
Accelerating voltage:	25000 V
1299.4Grid voltage:	92%
Guide wire 0:	0.04%
Extraction delay time:	400 nsec
Acquisition mass range:	4000 – 10000 Da
Number of laser shots:	200/spectrum
Laser intensity:	2911
Laser Rep Rate:	3.0 Hz
Calibration type:	Default
Calibration matrix:	2,5-Dihydroxybenzoic acid
Low mass gate:	500 Da
Digitizer start time:	39.704
Bin size:	2 nsec
Number of data points:	11493
Vertical scale:	200 mV
Vertical offset:	0%
Input bandwidth:	500 MHz
Sample well:	66
Plate ID:	PLACA1
Serial number:	2081
Instrument name:	Voyager-DE RP
Plate type filename:	C:\VOYAGER\100 well plate.pt
Lab name:	Servei Espectrometria de Masses
Absolute x-position:	27406.1
Absolute y-position:	17825.3
Relative x-position:	418.62
Relative y-position:	797.793
Shots in spectrum:	179
Source pressure:	3.526e-007
Mirror pressure:	9.302e-008
TC2 pressure:	0.01553
TIS gate width:	20
TIS flight length:	941.4


HO
H
N
O
(V**HArg**LPPP)3
N
H
O
O
O-P-O
OH
CGCGAATTCGCG

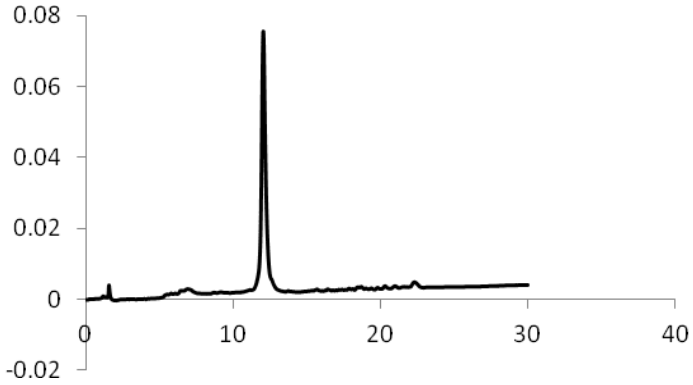
13a

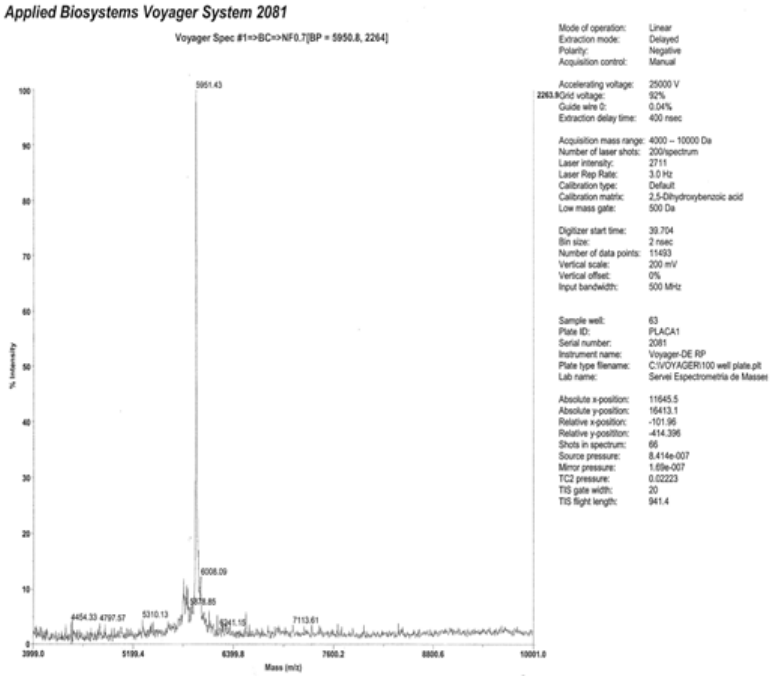

0.1
0.08
0.06
0.04
0.02
0
0
10
20
30
40

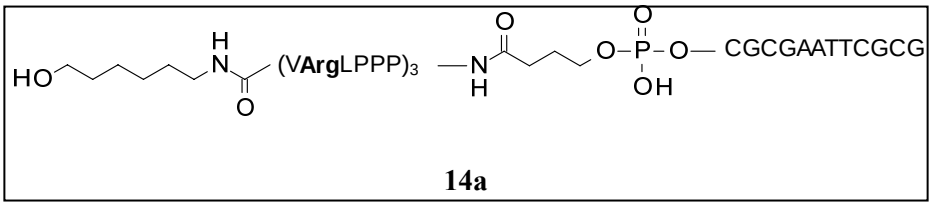

Applied Biosystems Voyager System 2081
Voyager Spec #1=>BC=>NF0.7[BP = 5922.3, 4783]
Mode of operation: Linear
Extraction mode: Delayed
Polarity: Negative
Acquisition control: Manual
Accelerating voltage: 25000 V
Grid voltage: 92%
Guide wire 0: 0.04%
Extraction delay time: 400 nsec
Acquisition mass range: 4000 – 10000 Da
Number of laser shots: 200/spectrum
Laser intensity: 2811
Laser Rep Rate: 3.0 Hz
Calibration type: Default
Calibration matrix: 2,5-Dihydroxybenzoic acid
Low mass gate: 500 Da
Digitizer start time: 39.704
Bin size: 2 nsec
Number of data points: 11493
Vertical scale: 200 mV
Vertical offset: 0%
Input bandwidth: 500 MHz
Sample well: 56
Plate ID: PLACA1
Serial number: 2081
Instrument name: Voyager-DE RP
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: Servei Espectrometria de Masses
Absolute x-position: 26563
Absolute y-position: 22124.3
Relative x-position: -424.48
Relative y-position: 216.786
Shots in spectrum: 47
Source pressure: 1.968e-007
Mirror pressure: 8.311e-008
TC2 pressure: 0.01443
TIS gate width: 20
TIS flight length: 941.4
4783.2
5920.80
5808.15
5771.48
5832.10
5430.74
5387.43
5366.05
4379.45
% Intensity
Mass (m/z)


HO
H
N
O
(V**HArg**LPPP)3
N
H
O
O
O–P–O—
OH
CGCGAATTCGCG

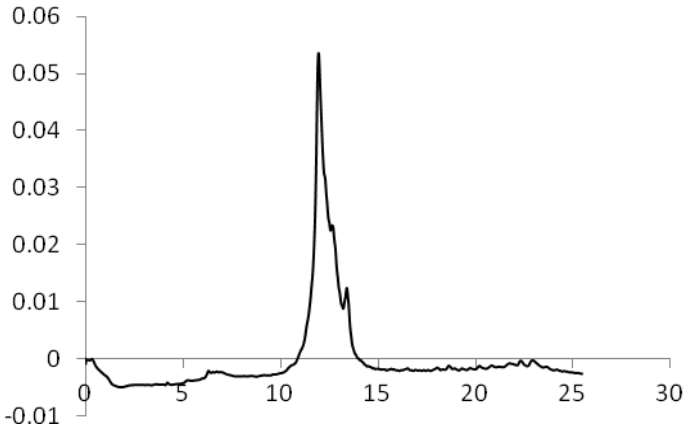
13b

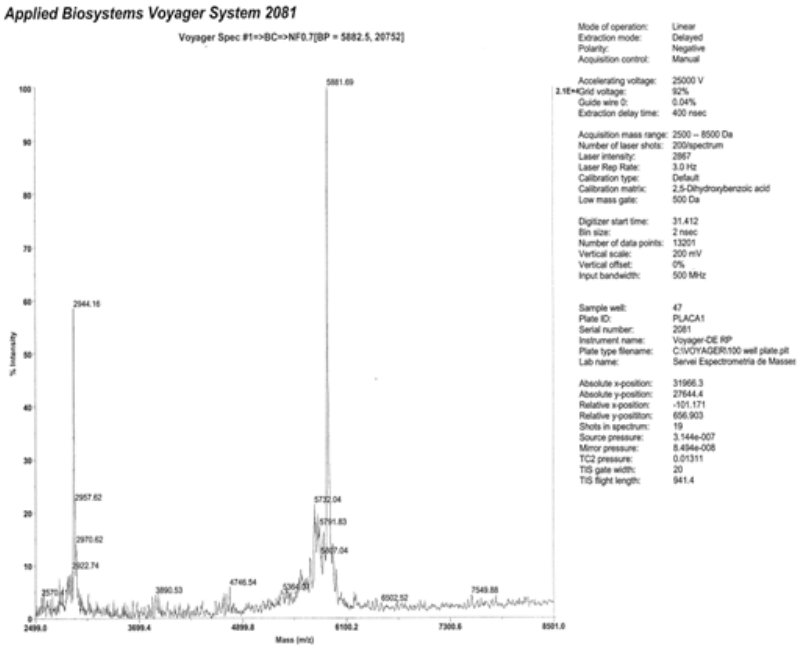

0.08
0.06
0.04
0.02
0
-0.02
0
10
20
30
40

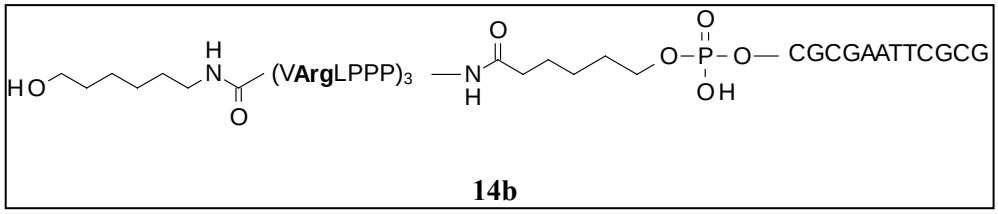

Applied Biosystems Voyager System 2081
Voyager Spec #1=>BC=>NF0.7[BP = 5950.8, 2264]
5951.43
6008.09
4454.33
4797.57
5310.13
7113.61
% Intensity
Mass (m/z)
Mode of operation: Linear
Extraction mode: Delayed
Polarity: Negative
Acquisition control: Manual
Accelerating voltage: 25000 V
Grid voltage: 92%
Guide wire 0: 0.04%
Extraction delay time: 400 nsec
Acquisition mass range: 4000 -- 10000 Da
Number of laser shots: 200/spectrum
Laser intensity: 2711
Laser Rep Rate: 3.0 Hz
Calibration type: Default
Calibration matrix: 2,5-Dihydroxybenzoic acid
Low mass gate: 500 Da
Digitizer start time: 39.704
Bin size: 2 nsec
Number of data points: 11493
Vertical scale: 200 mV
Vertical offset: 0%
Input bandwidth: 500 MHz
Sample well: 63
Plate ID: PLACA1
Serial number: 2081
Instrument name: Voyager-DE RP
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: Servei Espectrometria de Masses
Absolute x-position: 11645.5
Absolute y-position: 16413.1
Relative x-position: -101.96
Relative y-position: -414.396
Shots in spectrum: 66
Source pressure: 8.414e-007
Mirror pressure: 1.69e-007
TC2 pressure: 0.02223
TIS gate width: 20
TIS flight length: 941.4


HO
H
N
O
(V**Arg**LPPP)3
O
N
H
O
O
P
O
OH
CGCGAATTCGCG

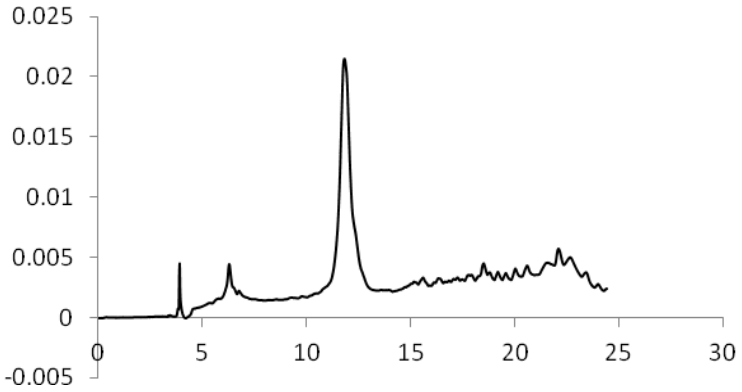
14a

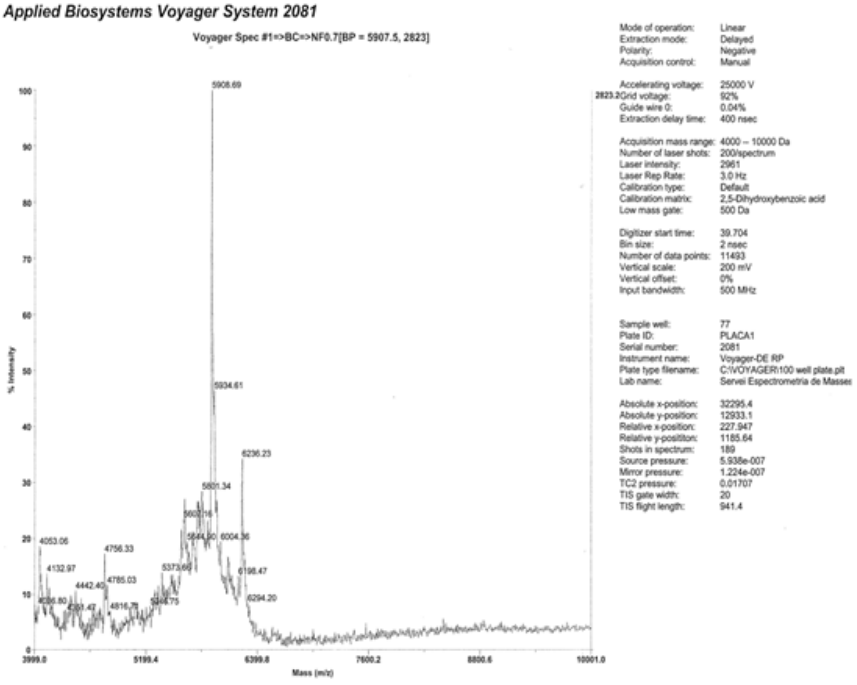

0.06
0.05
0.04
0.03
0.02
0.01
0
-0.01
0
5
10
15
20
25
30


Applied Biosystems Voyager System 2081
Voyager Spec #1=>BC=>NF0.7[BP = 5882.5, 20752]
5881.69
2944.16
2957.62
2970.62
2902.74
5732.04
5791.83
5847.04
3890.53
4746.54
6502.52
7549.88
% Intensity
Mass (m/z)
Mode of operation: Linear
Extraction mode: Delayed
Polarity: Negative
Acquisition control: Manual
Accelerating voltage: 25000 V
Grid voltage: 92%
Guide wire 0: 0.04%
Extraction delay time: 400 nsec
Acquisition mass range: 2500 – 8500 Da
Number of laser shots: 200/spectrum
Laser intensity: 2867
Laser Rep Rate: 3.0 Hz
Calibration type: Default
Calibration matrix: 2,5-Dihydroxybenzoic acid
Low mass gate: 500 Da
Digitizer start time: 31.412
Bin size: 2 nsec
Number of data points: 13201
Vertical scale: 200 mV
Vertical offset: 0%
Input bandwidth: 500 MHz
Sample well: 47
Plate ID: PLACA1
Serial number: 2081
Instrument name: Voyager-DE RP
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: Servei Espectrometria de Masses
Absolute x-position: 31966.3
Absolute y-position: 27644.4
Relative x-position: -101.171
Relative y-position: 696.903
Shots in spectrum: 19
Source pressure: 3.144e-007
Mirror pressure: 8.494e-008
TC2 pressure: 0.01311
TIS gate width: 20
TIS flight length: 941.4

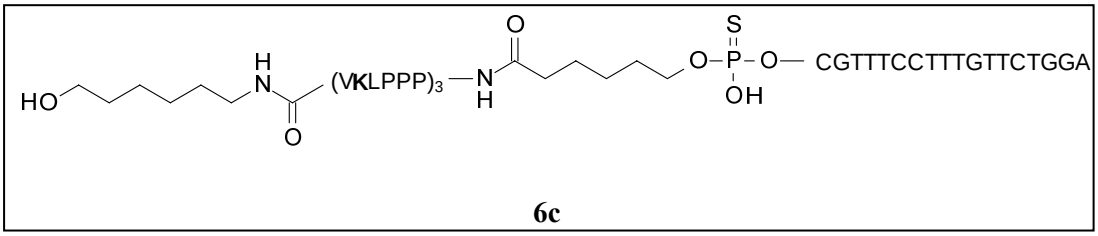

HO
H
N
O
(VArgLPPP)3
O
N
H
O
O
P
O
OH
O
CGCGAATTCGCG

14b

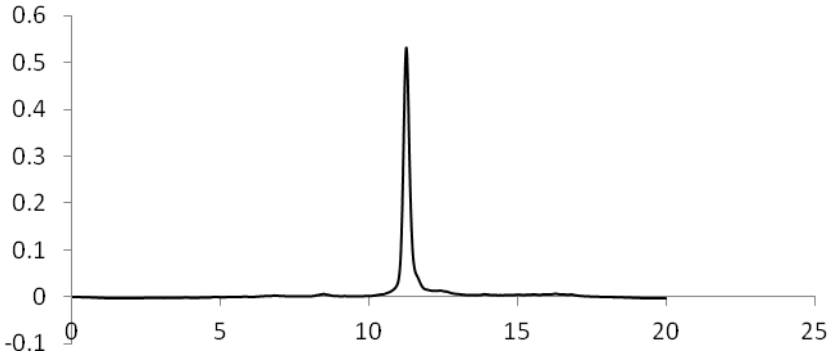

0.025
0.02
0.015
0.01
0.005
0
-0.005
0
5
10
15
20
25
30

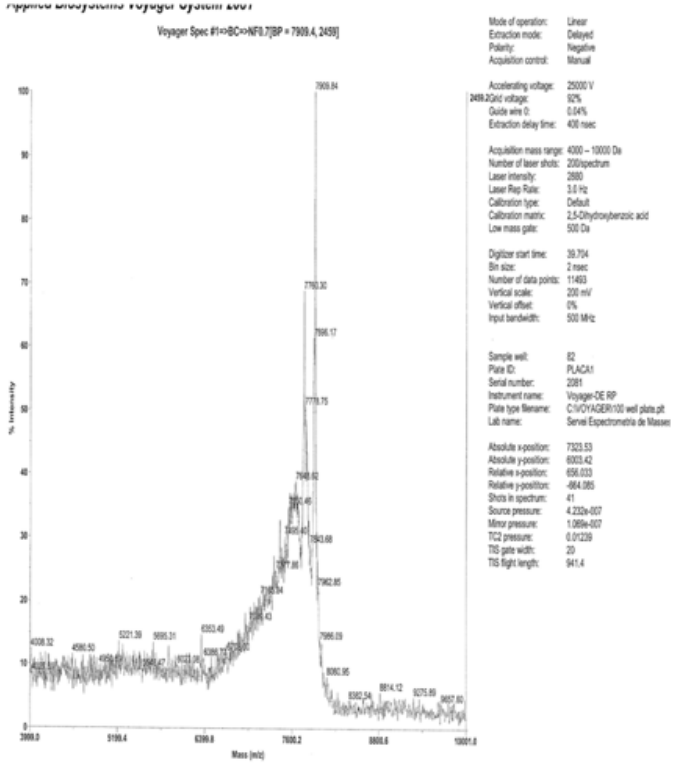

Applied Biosystems Voyager System 2081
Voyager Spec #1=>BC=>NF0.7[BP = 5907.5, 2823]
% Intensity
Mass (m/z)
5908.69
5934.61
6236.23
5601.34
6004.36
6198.47
6294.20
4053.06
4132.97
4756.33
4785.03
4442.40
5373.66
3999.0
5199.4
6399.8
7600.2
8800.6
10001.0
Mode of operation: Linear
Extraction mode: Delayed
Polarity: Negative
Acquisition control: Manual
Accelerating voltage: 25000 V
2823.2 Grid voltage: 92%
Guide wire 0: 0.04%
Extraction delay time: 400 nsec
Acquisition mass range: 4000 – 10000 Da
Number of laser shots: 200/spectrum
Laser intensity: 2961
Laser Rep Rate: 3.0 Hz
Calibration type: Default
Calibration matrix: 2,5-Dihydroxybenzoic acid
Low mass gate: 500 Da
Digitizer start time: 39.704
Bin size: 2 nsec
Number of data points: 11493
Vertical scale: 200 mV
Vertical offset: 0%
Input bandwidth: 500 MHz
Sample well: 77
Plate ID: PLACA1
Serial number: 2081
Instrument name: Voyager-DE RP
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: Servei Espectrometria de Masses
Absolute x-position: 32295.4
Absolute y-position: 12933.1
Relative x-position: 227.947
Relative y-posititon: 1185.64
Shots in spectrum: 189
Source pressure: 5.938e-007
Mirror pressure: 1.224e-007
TC2 pressure: 0.01707
TIS gate width: 20
TIS flight length: 941.4

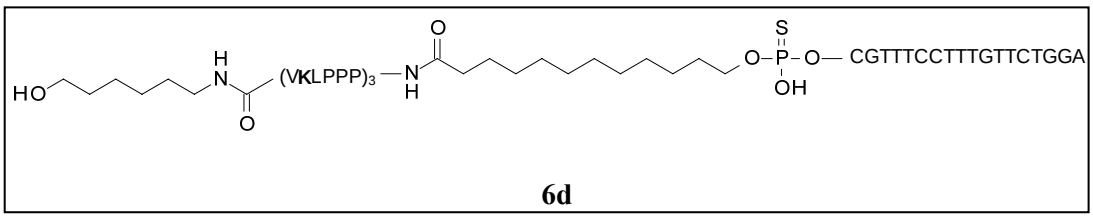
SAP-antisense phosphorothioate conjugates. HPLC chromatograms and MALDI-TOFF mass spectrometry spectra



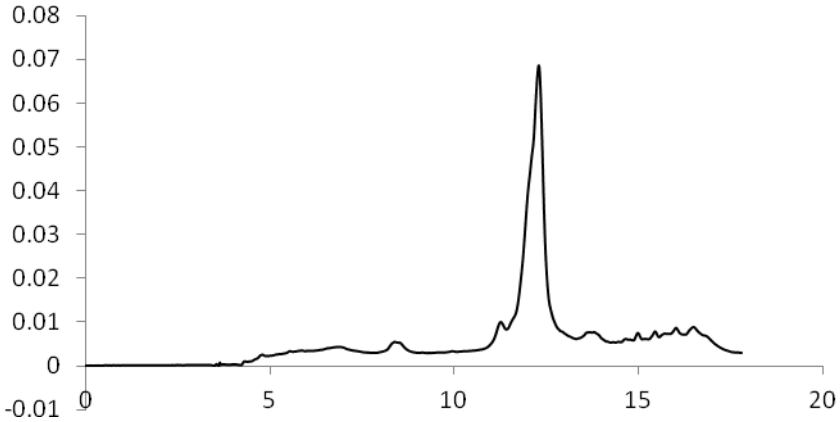
6c

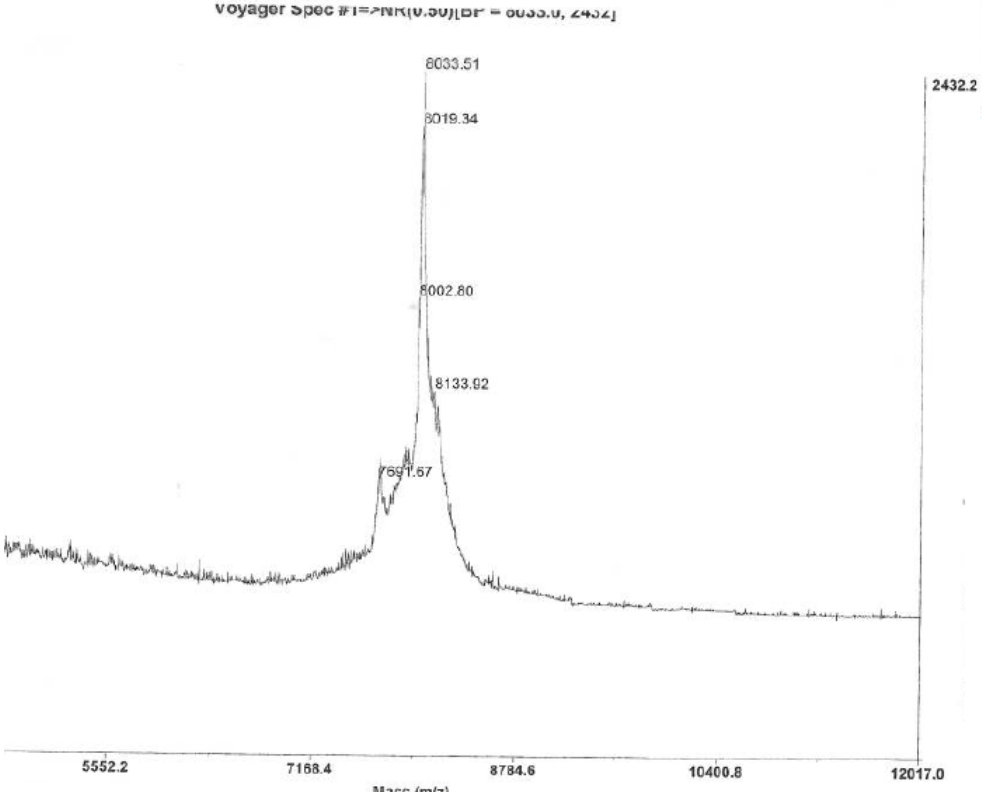


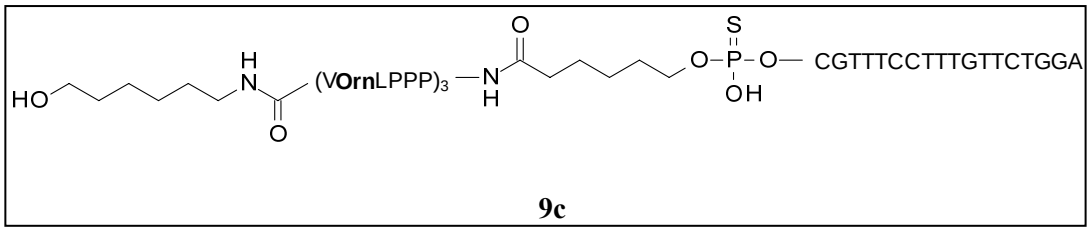



HO
H
N
O
(VKLPPP)3
N
H
O
O
S
O–P–O— CGTTTCCTTTGTTCTGGA
OH

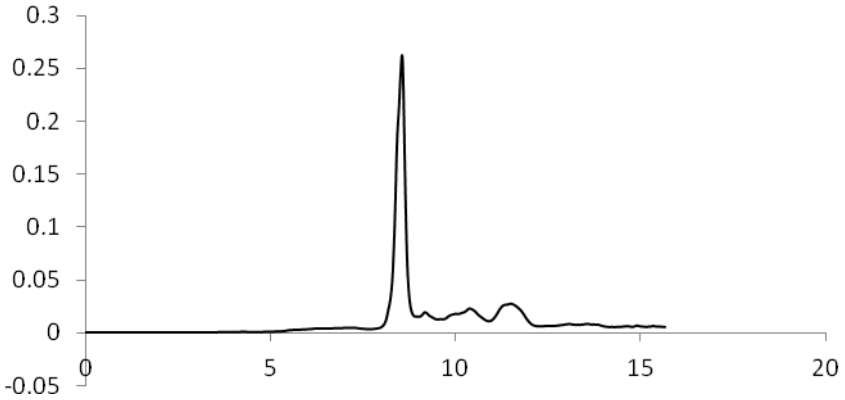
6d

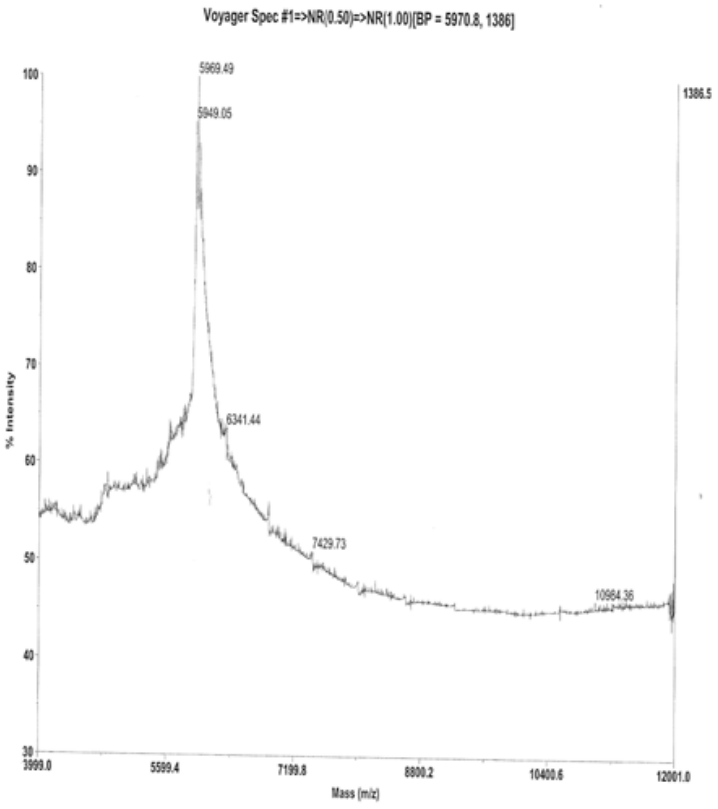

0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
-0.01
0
5
10
15
20

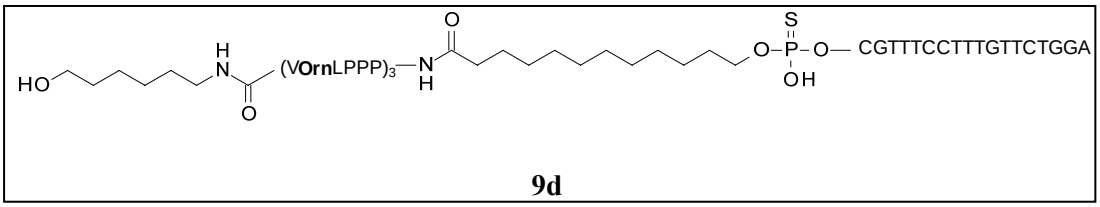

8033.51
8019.34
8002.80
8133.92
7991.67
2432.2
5552.2
7158.4
8784.6
10400.8
12017.0


HO
H
N
O
(V**Orn**LPPP)3
N
H
O
O
S
O–P–O
OH
CGTTTCCTTTGTTCTGGA

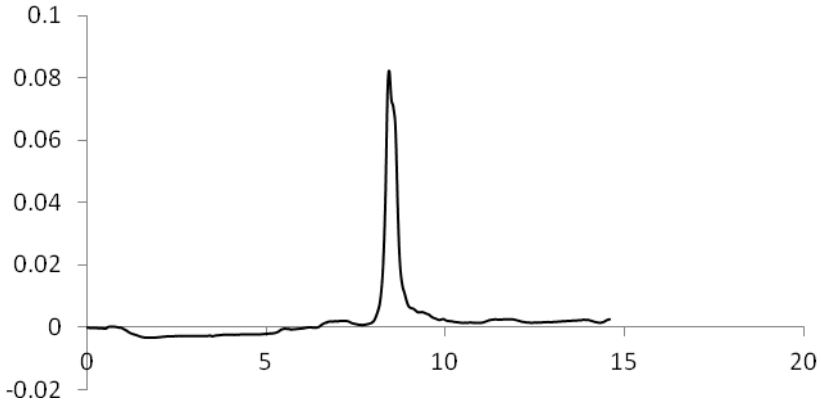
9c


0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
0
5
10
15
20


Voyager Spec #1=>NR(0.50)=>NR(1.00)[BP = 5970.8, 1386]
5969.49
5949.05
1386.5
6341.44
7429.73
10964.36
% Intensity
Mass (m/z)
3999.0
5599.4
7199.8
8800.2
10400.6
12001.0


HO
H
N
O
(V**Orn**LPPP)$_3$
N
H
O
O
S
O
P
O
OH
O
CGTTTCCTTTGTTCTGGA

9d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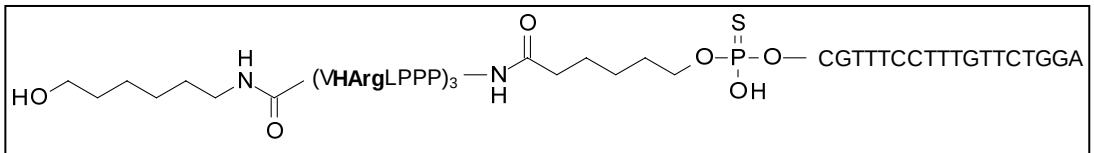
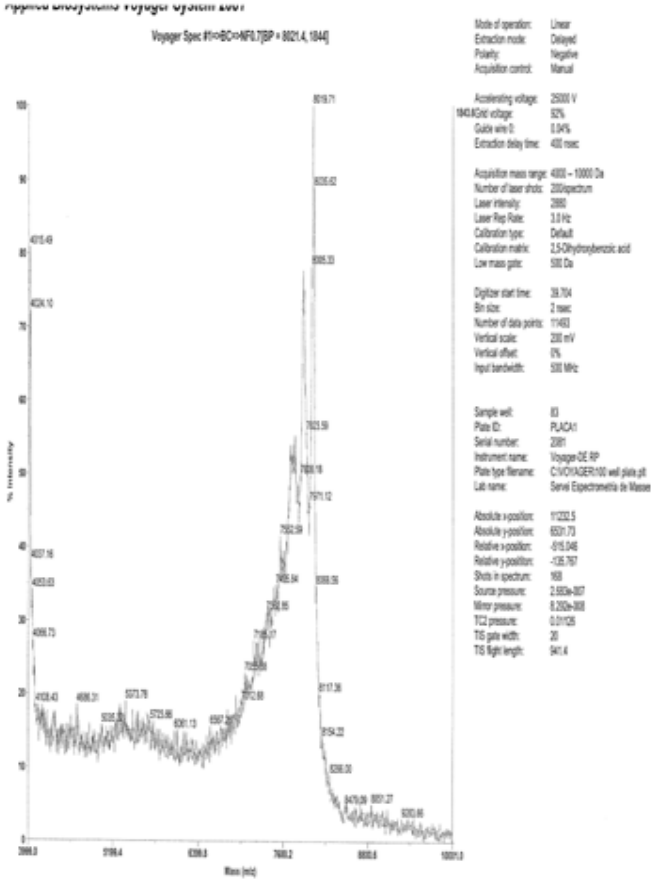
0.08
0.06
0.04
0.02
0
-0.02
0
5
10
15
20



13c



Mode of operation: Linear
Extraction mode: Delayed
Polarity: Negative
Acquisition control: Manual

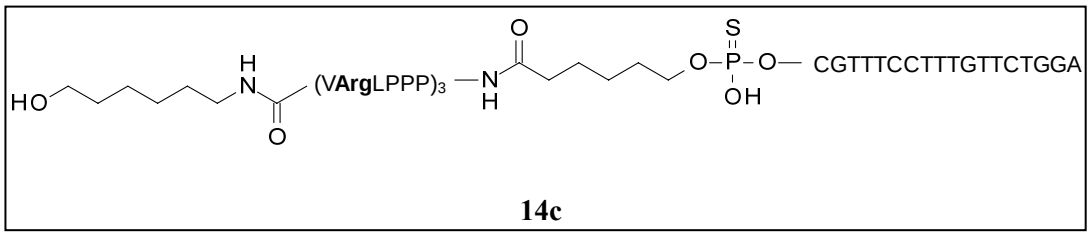
Accelerating voltage: 25000 V
Grid voltage: 92%
Guide wire 0: 0.04%
Extraction delay time: 400 nsec

Acquisition mass range: 4000 – 10000 Da
Number of laser shots: 200/spectrum
Laser intensity: 2880
Laser Rep Rate: 3.0 Hz
Calibration type: Default
Calibration matrix: 2,5-DHydroxybenzoic acid
Low mass gate: 500 Da

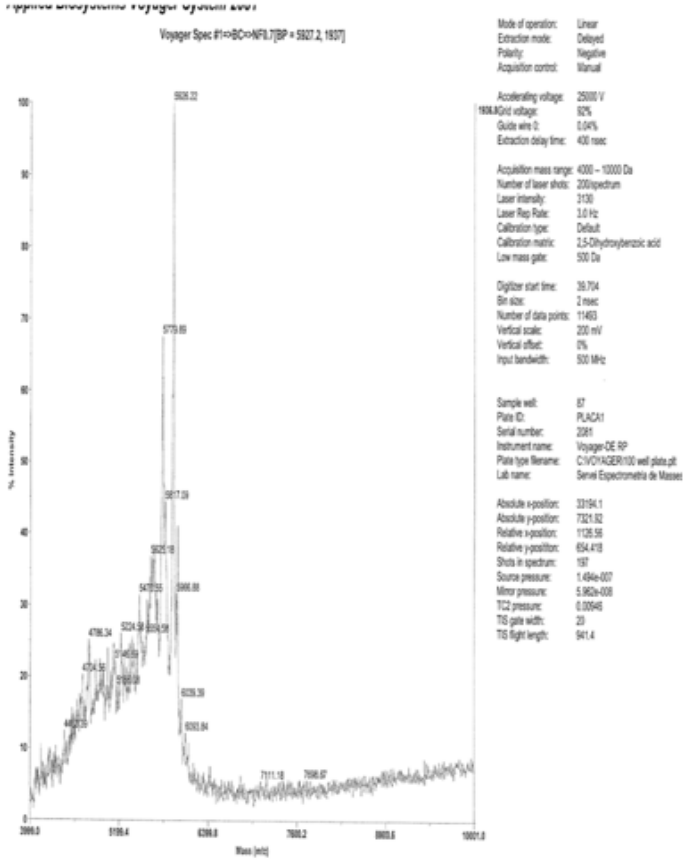
Digitizer start time: 39.704
Bin size: 2 nsec
Number of data points: 11493
Vertical scale: 200 mV
Vertical offset: 0%
Input bandwidth: 500 MHz

Sample well: B3
Plate ID: PLACA1
Serial number: 2081
Instrument name: Voyager-DE RP
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: Servei Espectrometria de Masses

Absolute x-position: 11232.5
Absolute y-position: 6521.73
Relative x-position: -615.048
Relative y-position: -135.767
Shots in spectrum: 168
Source pressure: 2.683e-007
Mirror pressure: 8.292e-008
TOF pressure: 0.01126
TIS gate width: 20
TIS flight length: 941.4



14c



Mode of operation: Linear
Extraction mode: Delayed
Polarity: Negative
Acquisition control: Manual

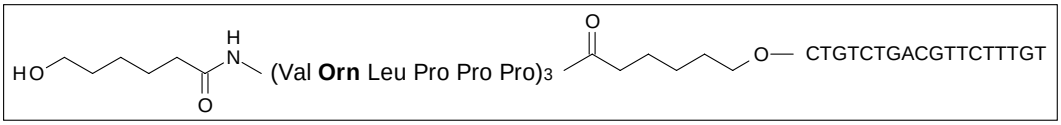
Accelerating voltage: 25000 V
Grid voltage: 92%
Guide wire 0: 0.04%
Extraction delay time: 400 nsec

Acquisition mass range: 4000 – 10000 Da
Number of laser shots: 200/spectrum
Laser intensity: 3130
Laser Rep Rate: 3.0 Hz
Calibration type: Default
Calibration matrix: 2,5-Dihydroxybenzoic acid
Low mass gate: 500 Da

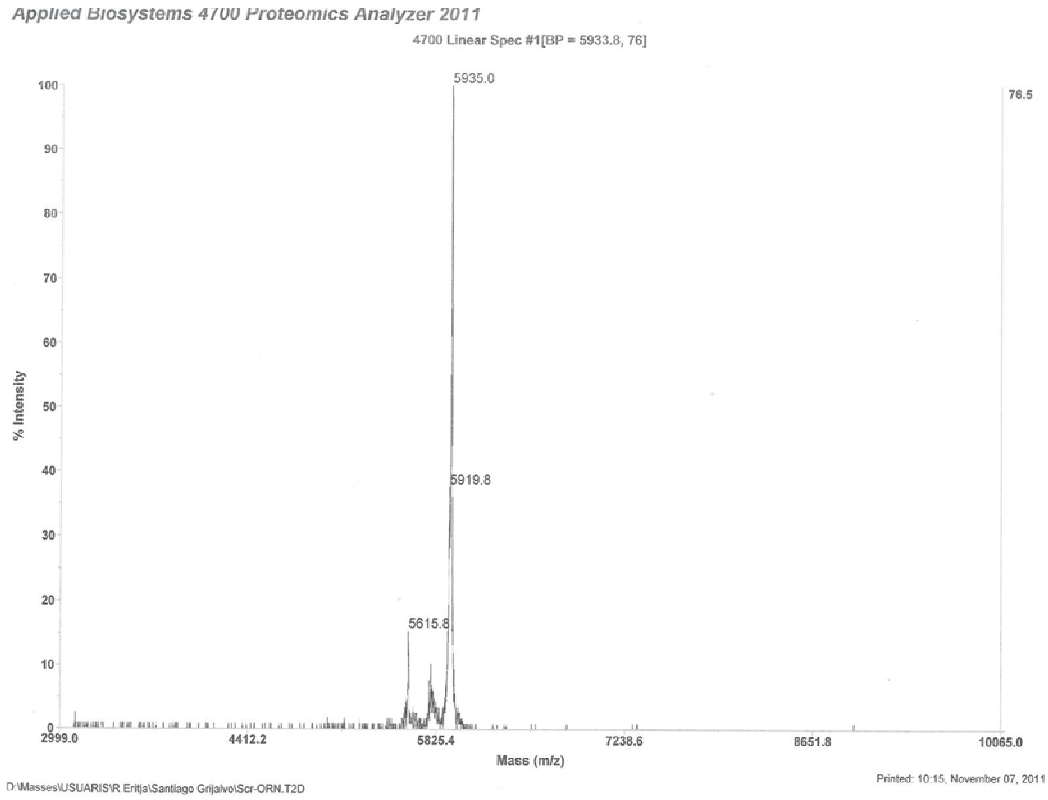
Digitizer start time: 39.704
Bin size: 2 nsec
Number of data points: 11493
Vertical scale: 200 mV
Vertical offset: 0%
Input bandwidth: 500 MHz

Sample well: 87
Plate ID: PLACA1
Serial number: 2261
Instrument name: Voyager-DE RP
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: Servei Espectrometria de Masses

Absolute x-position: 33194.1
Absolute y-position: 7321.92
Relative x-position: 1126.56
Relative y-position: 654.418
Shots in spectrum: 197
Source pressure: 1.494e-007
Mirror pressure: 5.962e-008
TC2 pressure: 0.00946
TIS gate width: 23
TIS flight length: 941.4


HO
H
N
O
(Val **Orn** Leu Pro Pro Pro)3
O
O
CTGTCTGACGTTCTTTGT

Scr-Orn


Applied Biosystems 4700 Proteomics Analyzer 2011
4700 Linear Spec #1[BP = 5933.8, 76]
5935.0
76.5
5919.8
5615.8
% Intensity
Mass (m/z)
2999.0
4412.2
5825.4
7238.6
8651.8
10065.0
D:\Masses\USUARIS\R Eritja\Santiago Grijalvo\Scr-ORN.T2D
Printed: 10:15, November 07, 2011

Polyacrylamide gel electrophoresis (SDS-PAGE)

Fig. 1S. Native SDS-PAGE gel shift assay to analyze the ability of SAP peptide to form complexes with unmodified oligonucleotide phosphorothioate (**wt)**. SAP peptide shows the highest propensity for the formation of the complex with the phosphorothioate oligonucleotide at a 10-fold molar excess of SAP

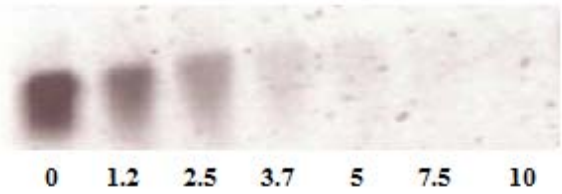
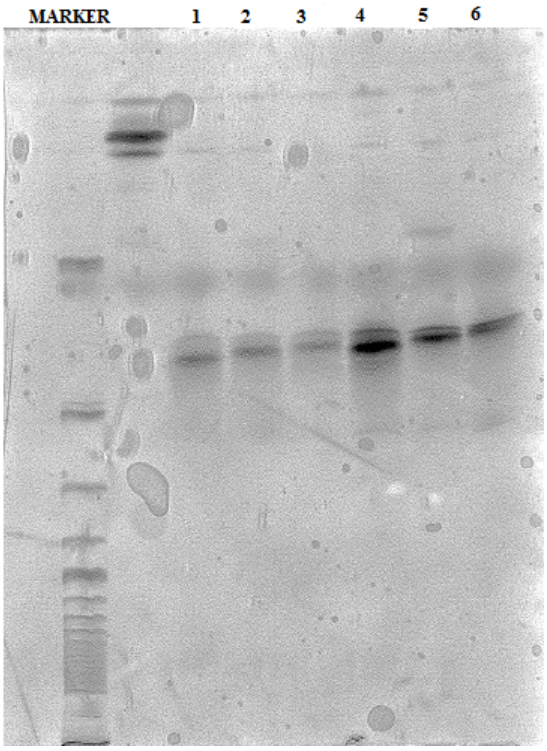


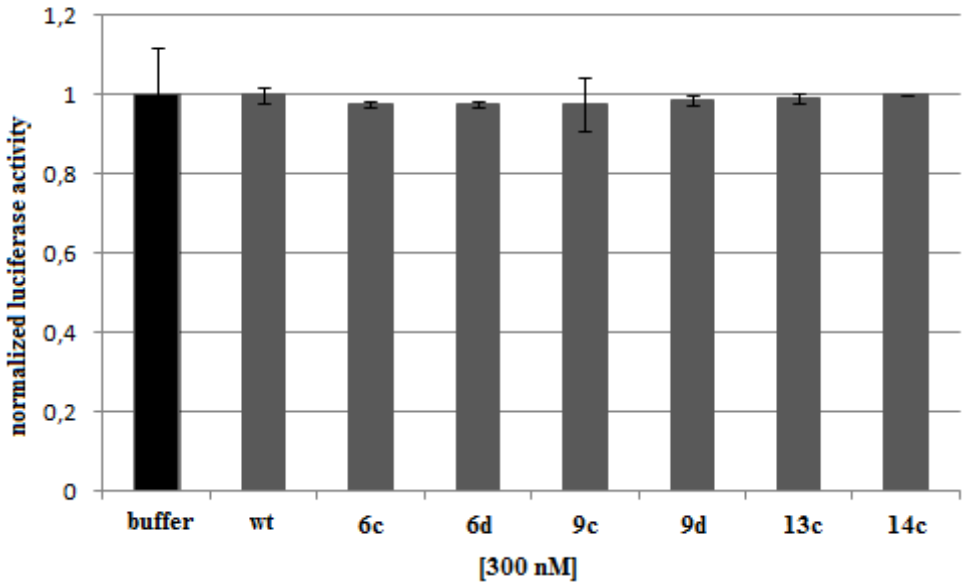
Fig. 2S 7M urea PAGE of SAP-antisense phosphorothioate conjugates



20 % Polyacrylamide, 8M urea gel electrophoresis of SAP-antisense phosphorothioate conjugates. The gel was stained with STAINS-ALL. Lane 1: SAP-antisense phosphorothioate **6c**; Lane 2: **6d**; Lane 3: **9c**; Lane 4: **9d**; Lane 5: **13c**; Lane 6: **14c**

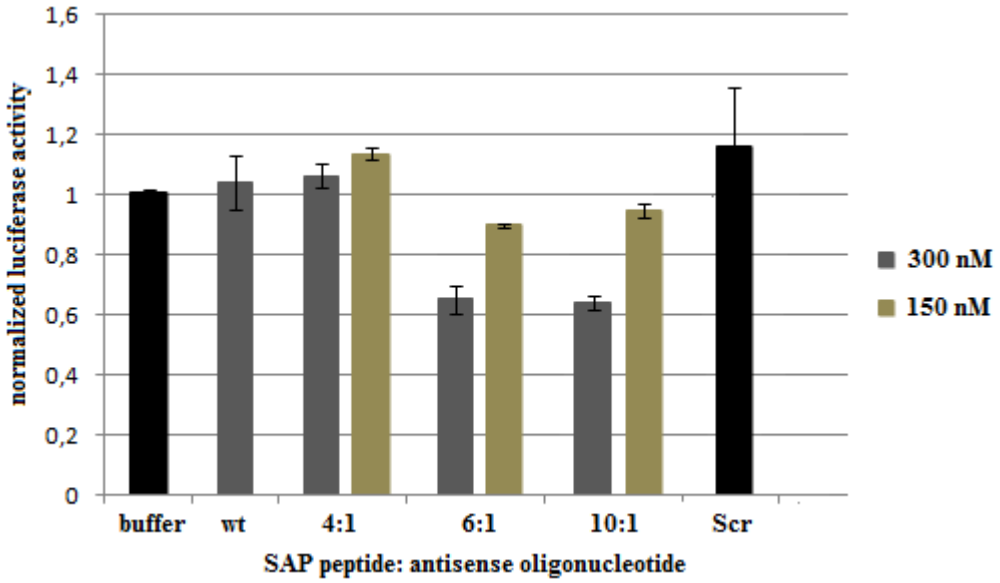
Cell Culture

Fig. 3S Gene-specific silencing activities of antisense phosphorothioate oligonucleotide conjugates without lipofectamine at 300 nM oligonucleotide concentration



Gene-specific silencing activities for unmodified phosphorothioate oligonucleotide (**wt**), and SAP-antisense phosphorothioate conjugates (**6c**, **15**, **9c**, **16, 13c** and **14c**; 300 nM per well) targeting the *Renilla* luciferase mRNA expressed in SH-SY5Y cells. Transfection of antisense oligonucleotides was carried out without using Lipofectamine 2000.

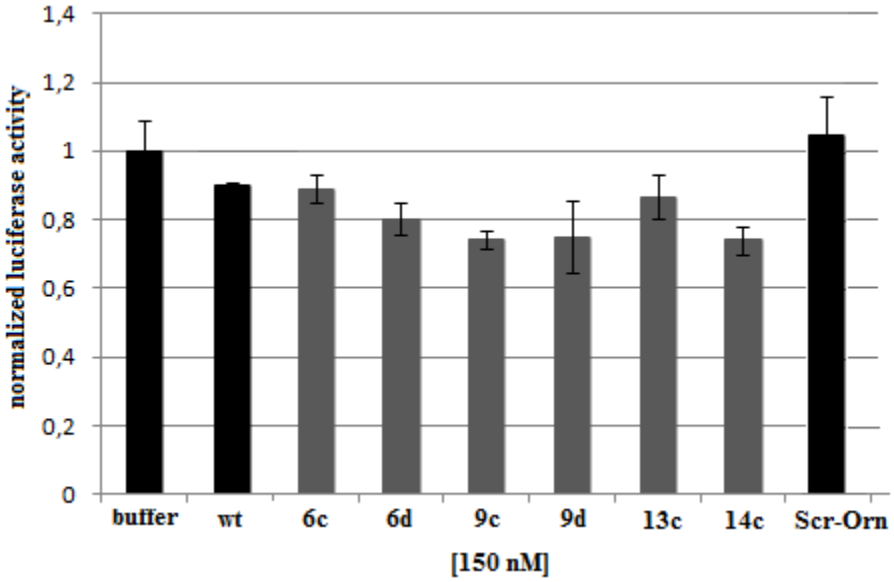
Fig. 4S Dose-response experiments of SAP: antisense phosphorothioate complexes at 150 nM and 300 nM oligonucleotide concentration



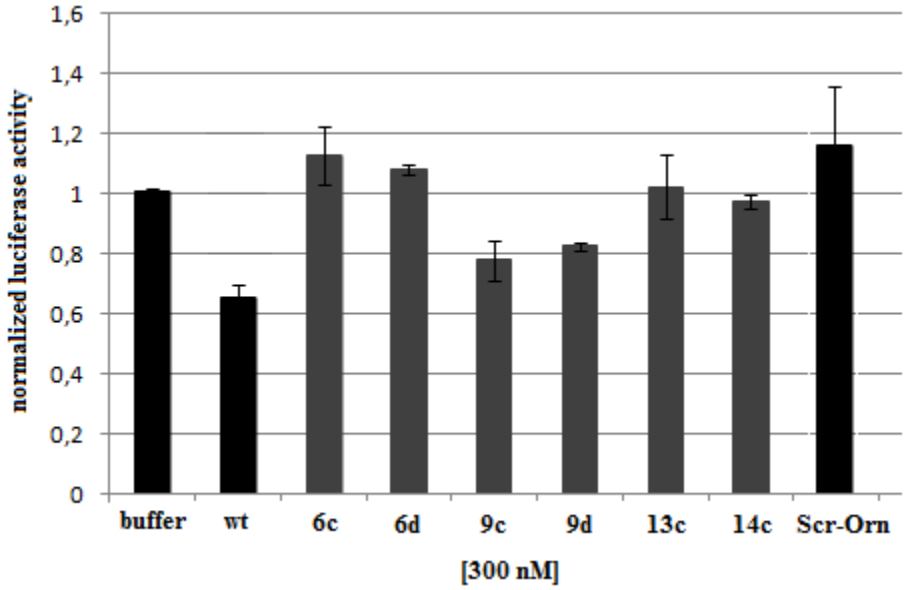
Gene-specific silencing activities for unmodified antisense oligonucleotide (**wt**) complexed with SAP peptide. The concentration of antisense oligonucleotide was 150 nM and 300 nM, respectively. The molar peptide: antisense oligonucleotide ratio used was 4:1, 6:1 and 10:1 targeting the *Renilla* luciferase mRNA expressed in SH-SY5Y cells. Transfection was carried out using SAP peptide as transfecting agent. The bar **wt** represents the value of the luciferase activity without using SAP peptide as transfecting agent. A scramble sequence combined with SAP peptide in a 6:1 M ratio gave no *Renilla* luciferase inhibition.

Fig. 5S Gene-specific silencing activities for antisense phosphorothioate oligonucleotide conjugates using SAP peptide as a transfecting agent at a molar ratio of 6:1 (SAP peptide / oligonucleotide).

A



B



Gene-specific silencing activities for phosphorothiote oligonucleotide conjugates (**wt**, **6c**, **6d**, **9c**, **9d**, **13c**, **14c** and **Scr-Orn**) targeting the *Renilla* luciferase mRNA expressed in SH-SY5Y cells. Transfection of antisense oligonucleotides was carried out using SAP peptide as transfecting agent in a 6-fold molar excess (peptide:SAP-antisense conjugate) (**A**) Experiment was performed at 150 nM oligonucleotide concentration; (**B**) Experiment was performed at 300 nM oligonucleotide concentration. Scrambled sequence (**Scr-Orn**) gave no *Renilla* luciferase inhibition.